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Commissioner for Patents
Washington, D.C. 20231

CONTINUATION-IN-PART APPLICATION

TRANSMITTAL

Sir:

Transmitted herewith for filing is a **Continuation-in-Part** of U.S. Application No. not yet assigned, filed June 15, 2000, which is a continuation-in-part of U.S. Application No. 09/357,743, filed July 20, 1999, which is a continuation-in-part of U.S. Application No. 09/357,024, filed July 19, 1999, which claims the benefit of U.S. Provisional Application 60/093,484, filed July 20, 1998.

Inventor(s): Vincent P. Stanton, Jr.

Title: **GENE SEQUENCE VARIANCES IN GENES RELATED TO FOLATE METABOLISM HAVING UTILITY IN DETERMINING THE TREATMENT OF DISEASE**

I. PAPERS ENCLOSED HEREWITH FOR FILING UNDER 37 CFR § 1.53(b):

195 Page(s) of Written Description

20 Page(s) Claims

1 Page(s) Abstract

___ Other:

2 Sheets of Drawings X Informal _____ Formal

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(37 C.F.R. §1.10)

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Respectfully submitted,

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CONTINUATION-IN-PART APPLICATION

UNDER 37 CFR § 1.53(B)

TITLE: GENE SEQUENCE VARIANCES IN GENES RELATED TO FOLATE METABOLISM HAVING UTILITY IN DETERMINING THE TREATMENT OF DISEASE

APPLICANT(S): VINCENT P. STANTON, JR.

Correspondence Enclosed:

Continuation-in-Part Transmittal (3 pgs); Cover Sheet (1pg); Description (195 pgs); Claims (20 pgs); Abstract (1 pg); Drawings (2 pgs); and Return Postcard

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DESCRIPTION

GENE SEQUENCE VARIANCES IN GENES RELATED TO FOLATE METABOLISM HAVING UTILITY IN DETERMINING THE TREATMENT OF DISEASE

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Application No. not yet assigned, filed June 15, 2000, which is a continuation-in-part of Stanton, U.S. Application 09/357,743, filed July 20, 1999, entitled GENE SEQUENCE VARIANCES WITH UTILITY IN DETERMINING THE TREATMENT OF DISEASE which is a CIP of Stanton, U.S. Application Serial No. 09/357,024, filed July 19, 1999, entitled GENE SEQUENCE VARIANCES WITH UTILITY IN DETERMINING THE TREATMENT OF DISEASE, which claims the benefit of Stanton, U.S. Provisional Application 60/093,484, filed July 20, 1998, entitled GENE SEQUENCE VARIANCES WITH UTILITY IN DETERMINING THE TREATMENT OF DISEASE, which are all hereby incorporated by reference in their entireties including drawings and tables.

BACKGROUND OF THE INVENTION

This application concerns the field of mammalian therapeutics and the selection of therapeutic regimens utilizing host genetic information, including gene sequence variances within the human genome in human populations.

The rate of approval of new drugs that enter human clinical trials is less than 20%, despite demonstrated efficacy of said new drugs in preclinical models of human disease. In some instances the low response rate in humans is due to genetic heterogeneity in the drug target or the pathway mediating the action of the drug. Identification of the genetic causes of variable drug response would allow more rational clinical development of drugs. Further, many drugs or other treatments approved for use in humans are known to have highly variable safety and efficacy in different individuals. A consequence of such variability is that a given drug or other treatment may be highly effective in one individual, and ineffective or not well tolerated in another individual. Thus, administration of such a drug to

an individual in whom the drug would be ineffective would result in wasted cost and time during which the patient's condition may significantly worsen. Also, administration of a drug to an individual in whom the drug would not be tolerated could result in a direct worsening of the patient's condition and could even result in the patient's death.

For some drugs, up to 99% of the measurable variation in selected pharmacokinetic parameters has been shown to be inherited, or associated with genetic factors. Studies have also demonstrated a significant genetic component to pharmacodynamic variation. For a limited number of drugs, discrete gene sequence variances have been identified in specific genes that are involved in drug action, and these variances have been shown to account for the variable efficacy or safety of the drug in different individuals.

SUMMARY OF THE INVENTION

The present invention is concerned generally with the field of treatment of diseases and conditions in mammals, particularly in humans. It is concerned with the genetic basis of inter-patient variation in response to therapy, including drug therapy. Specifically, this invention describes the identification of gene sequence variances useful in the field of therapeutics for optimizing efficacy and safety of drug therapy for specific diseases or conditions and for establishing diagnostic tests useful for improving the development and use of pharmaceutical products in the clinic. Methods for identifying genetic variances and determining their utility in the selection of optimal therapy for specific patients are also described, along with probes and related materials which are useful, for example, in identifying the presence of a particular gene sequence variance in cells of an individual. The genes involved in the present invention are those listed in a pathway, gene table, list or example herein.

The inventors have determined that the identification of gene sequence variances within genes that may be involved in drug action is important for determining whether genetic variances account for variable drug efficacy and safety and for determining whether a given drug or other therapy may be safe and effective in an individual patient. Provided in this invention are identifications of genes and sequence variances which can be useful in connection with predicting differences in response to treatment and selection of appropriate treatment of a disease or condition. Such genes and variances have utility in

pharmacogenetic association studies and diagnostic tests to improve the use of certain drugs or other therapies including, but not limited to, the drug classes and specific drugs identified in the 1999 Physicians' Desk Reference (53rd edition), Medical Economics Data, 1998, or the 1995 United States Pharmacopeia XXIII National Formulary XVIII, Interpharm Press, 1994, or other sources as described below.

The terms "disease" or "condition" are commonly recognized in the art and designate the presence of signs and/or symptoms in an individual or patient that are generally recognized as abnormal. Diseases or conditions may be diagnosed and categorized based on pathological changes. Signs may include any objective evidence of a disease such as changes that are evident by physical examination of a patient or the results of diagnostic tests which may include, among others, laboratory tests to determine the presence of variances or variant forms of certain genes in a patient. Symptoms are subjective evidence of disease or a patients condition – i.e. the patients perception of an abnormal condition that differs from normal function, sensation, or appearance, which may include, without limitations, physical disabilities, morbidity, pain, and other changes from the normal condition experienced by an individual. Various diseases or conditions include, but are not limited to, those categorized in standard textbooks of medicine including, without limitation, textbooks of nutrition, allopathic, homeopathic, and osteopathic medicine. In certain aspects of this invention, the disease or condition is selected from the group consisting of the types of diseases listed in standard texts such as Harrison's Principles of Internal Medicine (14th Ed) by Anthony S. Fauci, Eugene Braunwald, Kurt J. Isselbacher, et al. (Editors), McGraw Hill, 1997, or Robbins Pathologic Basis of Disease (6th edition) by Ramzi S. Cotran, Vinay Kumar, Tucker Collins & Stanley L. Robbins, W B Saunders Co., 1998, or the Diagnostic and Statistical Manual of Mental Disorders: Dsm-IV (4th Ed), American Psychiatric Press, 1994 or other texts described below.

In connection with the methods of this invention, unless otherwise indicated, the term "suffering from a disease or condition" means that a person is either presently subject to the signs and symptoms, or is more likely to develop such signs and symptoms than a normal person in the population. Thus, for example, a person suffering from a condition can include a developing fetus, a person subject to a treatment or environmental condition which enhances the likelihood of developing the signs or symptoms of a condition, or a person who is being given or will be given a treatment which increase the likelihood of the person

developing a particular condition. For example, tardive dyskinesia is associated with long-term use of anti-psychotics; gastrointestinal symptoms, alopecia and bone marrow suppression are associated with cancer chemotherapeutic regimens, and immunosuppression is associated with agents to limit graft rejection following transplantation. Thus, methods of the present invention which relate to treatments of patients (e.g., methods for selecting a treatment, selecting a patient for a treatment, and methods of treating a disease or condition in a patient) can include primary treatments directed to a presently active disease or condition, secondary treatments which are intended to cause a biological effect relevant to a primary treatment, and prophylactic treatments intended to delay, reduce, or prevent the development of a disease or condition, as well as treatments intended to cause the development of a condition different from that which would have been likely to develop in the absence of the treatment.

The term "therapy" refers to a process which is intended to produce a beneficial change in the condition of a mammal, e.g., a human, often referred to as a patient. A beneficial change can, for example, include one or more of: restoration of function, reduction of symptoms, limitation or retardation of progression of a disease, disorder, or condition or prevention, limitation or retardation of deterioration of a patient's condition, disease or disorder. Such therapy can involve, for example, nutritional modifications, administration of radiation, administration of a drug, behavioral modifications and combinations of these, among others.

The term "drug" as used herein refers to a chemical entity or biological product, or combination of chemical entities or biological products, administered to a person to treat or prevent or control a disease or condition. The chemical entity or biological product is preferably, but not necessarily a low molecular weight compound, but may also be a larger compound, for example, an oligomer of nucleic acids, amino acids, or carbohydrates including without limitation proteins, oligonucleotides, ribozymes, DNAzymes, glycoproteins, lipoproteins, and modifications and combinations thereof. A biological product is preferably a monoclonal or polyclonal antibody or fragment thereof such as a variable chain fragment cells; or an agent or product arising from recombinant technology, such as, without limitation, a recombinant protein, recombinant vaccine, or DNA construct developed for therapeutic, e.g., human therapeutic, use. The term "drug" may include, without limitation, compounds that are approved for sale as pharmaceutical products by

government regulatory agencies (e.g., U.S. Food and Drug Administration (USFDA or FDA), European Medicines Evaluation Agency (EMEA), and a world regulatory body governing the Internation Conference of Harmonization (ICH) rules and guidelines), compounds that do not require approval by government regulatory agencies, food additives or supplements including compounds commonly characterized as vitamins, natural products, and completely or incompletely characterized mixtures of chemical entities including natural compounds or purified or partially purified natural products. The term "drug" as used herein is synonymous with the terms "medicine", "pharmaceutical product", or "product". Most preferably the drug is approved by a government agency for treatment of a specific disease or condition.

A "low molecular weight compound" has a molecular weight <5,000 Da, more preferably <2500 Da, still more preferably <1000 Da, and most preferably <700 Da.

Those familiar with drug use in medical practice will recognize that regulatory approval for drug use is commonly limited to approved indications, such as to those patients afflicted with a disease or condition for which the drug has been shown to be likely to produce a beneficial effect in a controlled clinical trial. Unfortunately, it has generally not been possible with current knowledge to predict which patients will have a beneficial response, with the exception of certain diseases such as bacterial infections where suitable laboratory methods have been developed. Likewise, it has generally not been possible to determine in advance whether a drug will be safe in a given patient. Regulatory approval for the use of most drugs is limited to the treatment of selected diseases and conditions. The descriptions of approved drug usage, including the suggested diagnostic studies or monitoring studies, and the allowable parameters of such studies, are commonly described in the "label" or "insert" which is distributed with the drug. Such labels or inserts are preferably required by government agencies as a condition for marketing the drug and are listed in common references such as the Physicians Desk Reference (PDR). These and other limitations or considerations on the use of a drug are also found in medical journals, publications such as pharmacology, pharmacy or medical textbooks including, without limitation, textbooks of nutrition, allopathic, homeopathic, and osteopathic medicine.

Many widely used drugs are effective in a minority of patients receiving the drug, particularly when one controls for the placebo effect. For example, the PDR shows that about 45% of patients receiving Cognex (tacrine hydrochloride) for Alzheimer's disease

show no change or minimal worsening of their disease, as do about 68% of controls (including about 5% of controls who were much worse). About 58% of Alzheimer's patients receiving Cognex were minimally improved, compared to about 33% of controls, while about 2% of patients receiving Cognex were much improved compared to about 1% of controls. Thus a tiny fraction of patients had a significant benefit. Response to many cancer chemotherapy drugs is even worse. For example, 5-fluorouracil is standard therapy for advanced colorectal cancer, but only about 20-40% of patients have an objective response to the drug, and, of these, only 1-5% of patients have a complete response (complete tumor disappearance; the remaining patients have only partial tumor shrinkage). Conversely, up to 20-30% of patients receiving 5-FU suffer serious gastrointestinal or hematopoietic toxicity, depending on the regimen.

Thus, in a first aspect, the invention provides a method for selecting a treatment for a patient suffering from a disease or condition by determining whether or not a gene or genes in cells of the patient (in some cases including both normal and disease cells, such as cancer cells) contain at least one sequence variance which is indicative of the effectiveness of the treatment of the disease or condition. The gene is one specified herein, in particular one listed in a Table or list herein. Preferably the at least one variance includes a plurality of variances which may provide a haplotype or haplotypes. Preferably the joint presence of the plurality of variances is indicative of the potential effectiveness of the treatment in a patient having such plurality of variances. The plurality of variances may each be indicative of the potential effectiveness of the treatment, and the effects of the individual variances may be independent or additive, or the plurality of variances may be indicative of the potential effectiveness if at least 2, 3, 4, or more appear jointly. The plurality of variances may also be combinations of these relationships. The plurality of variances may include variances from one, two, three or more gene loci.

In a related aspect, the invention concerns a method for providing a correlation between a patient genotype and effectiveness of a treatment, by determining the presence or absence of a particular known variance or variances in cells of a patient for a gene of this invention, and providing a result indicating the expected effectiveness of a treatment for a disease or condition. The result may be formulated by comparing the genotype of the patient with a list of variances indicative of the effectiveness of a treatment, e.g., administration of a

drug described herein. The determination may be by methods as described herein or other methods known to those skilled in the art.

In some cases, the selection of a method of treatment, i.e., a therapeutic regimen, may incorporate selection of one or more from a plurality of medical therapies. Thus, the selection may be the selection of a method or methods which is/are more effective or less effective than certain other therapeutic regimens (with either having varying safety parameters). Likewise or in combination with the preceding selection, the selection may be the selection of a method or methods which is safer than certain other methods of treatment in the patient.

The selection may involve either positive selection or negative selection or both, meaning that the selection can involve a choice that a particular method would be an appropriate method to use and/or a choice that a particular method would be an inappropriate method to use. Thus, in certain embodiments, the presence of the at least one variance is indicative that the treatment will be effective or otherwise beneficial (or more likely to be beneficial) in the patient. Stating that the treatment will be effective means that the probability of beneficial therapeutic effect is greater than in a person not having the appropriate presence or absence of particular variances. In other embodiments, the presence of the at least one variance is indicative that the treatment will be ineffective or contra-indicated for the patient. For example, a treatment may be contra-indicated if the treatment results, or is more likely to result, in undesirable side effects, or an excessive level of undesirable side effects. A determination of what constitutes excessive side-effects will vary, for example, depending on the disease or condition being treated, the availability of alternatives, the expected or experienced efficacy of the treatment, and the tolerance of the patient. As for an effective treatment, this means that it is more likely that a desired effect will result from the treatment administration in a patient with a particular variance or variances than in a patient who has a different variance or variances. Also in preferred embodiments, the presence of the at least one variance is indicative that the treatment is effective but results in undesirable effects or outcomes, e.g., has undesirable side-effects.

In reference to response to a treatment, the term "tolerance" refers to the ability of a patient to accept a treatment, based, e.g., on deleterious effects and/or effects on lifestyle. Frequently, the term principally concerns the patients perceived magnitude of deleterious effects such as nausea, weakness, dizziness, and diarrhea, among others. Such experienced

effects can, for example, be due to general or cell-specific toxicity, activity on non-target cells, cross-reactivity on non-target cellular constituents (non-mechanism based), and/or side-effects of activity on the target cellular subsitutuent (mechanism based), or the cause of toxicity may not be understood. In any of these circumstances one may identify an association between the undesirable effects and variances in specific genes.

Adverse responses to drugs constitute a major medical problem, as shown in two recent meta-analyses (Lazarou, J. et al, Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies, JAMA 279:1200-1205, 1998; Bonn, Adverse drug reactions remain a major cause of death, Lancet 351:1183, 1998). An estimated 2.2 million hospitalized patients in the United Stated had serious adverse drug reactions in 1994, with an estimated 106,000 deaths (Lazarou et al.). To the extent that some of these adverse events are due to genetically encoded biochemical diversity among patients in pathways that effect drug action, the identification of variances that are predictive of such effects will allow for more effective and safer drug use.

In embodiments of this invention, the variance or variant form or forms of a gene is/are associated with a specific response to a drug. The frequency of a specific variance or variant form of the gene may correspond to the frequency of an efficacious response to administration of a drug. Alternatively, the frequency of a specific variance or variant form of the gene may correspond to the frequency of an adverse event resulting from administration of a drug. Alternatively the frequency of a specific variance or variant form of a gene may not correspond closely with the frequency of a beneficial or adverse response, yet the variance may still be useful for identifying a patient subset with high response or toxicity incidence because the variance may account for only a fraction of the patients with high response or toxicity. Preferably, the drug will be effective in more than 20% of individuals with one or more specific variances or variant forms of the gene, more preferably in 40% and most preferably in >60%. In other embodiments, the drug will be toxic or create clinically unacceptable side effects in more than 10% of individuals with one or more variances or variant forms of the gene, more preferably in >30%, more preferably in >50%, and most preferably in >70% or in more than 90%.

Also in other embodiments, the method of selecting a treatment includes eliminating a treatment, where the presence or absence of the at least one variance is indicative that the treatment will be ineffective or contra-indicated. In other preferred embodiments, in cases in

which undesirable side-effects may occur or are expected to occur from a particular therapeutic treatment, the selection of a method of treatment can include identifying both a first and second treatment, where the first treatment is effective to treat the disease or condition, and the second treatment reduces a deleterious effect of the first treatment.

The phrase "eliminating a treatment" refers to removing a possible treatment from consideration, e.g., for use with a particular patient based on the presence or absence of a particular variance(s) in one or more genes in cells of that patient, or to stopping the administration of a treatment which was in the course of administration.

Usually, the treatment will involve the administration of a compound preferentially active in patients with a form or forms of a gene, where the gene is one identified herein. The administration may involve a combination of compounds. Thus, in preferred embodiments, the method involves identifying such an active compound or combination of compounds, where the compound is less active or is less safe or both when administered to a patient having a different form of the gene. In preferred embodiments, the compound is a compound in a drug class identified in the 1999 Physicians' Desk Reference (53rd edition), Medical Economics Data, 1998, the PharmaProjects database, the IMS database or identified herein, e.g., in an exemplary drug table herein (see, e.g., Examples 6, 8, and 9 and Tables 7 and 9 herein).

Also in preferred embodiments, the method of selecting a treatment involves selecting a method of administration of a compound, combination of compounds, or pharmaceutical composition, for example, selecting a suitable dosage level and/or frequency of administration, and/or mode of administration of a compound. The method of administration can be selected to provide better, preferably maximum therapeutic benefit. In this context, "maximum" refers to an approximate local maximum based on the parameters being considered, not an absolute maximum.

Also in this context, a "suitable dosage level" refers to a dosage level which provides a therapeutically reasonable balance between pharmacological effectiveness and deleterious effects. Often this dosage level is related to the peak or aveage serum levels resulting from administration of a drug at the particular dosage level.

Similarly, a "frequency of administration" refers to how often in a specified time period a treatment is administered, e.g., once, twice, or three times per day, every other day, once per week, etc. For a drug or drugs, the frequency of administration is generally selected

to achieve a pharmacologically effective average or peak serum level without excessive deleterious effects (and preferably while still being able to have reasonable patient compliance for self-administered drugs). Thus, it is desirable to maintain the serum level of the drug within a therapeutic window of concentrations for the greatest percentage of time possible without such deleterious effects as would cause a prudent physician to reduce the frequency of administration for a particular dosage level.

A particular gene or genes can be relevant to more than one disease or condition, for example, the gene or genes can have a role in the initiation, development, course, treatment, treatment outcomes, or health-related quality of life outcomes of a number of different diseases, disorders, or conditions. Thus, in preferred embodiments, the disease or condition or treatment of the disease or condition is any which involves a particular gene. Preferably the gene is a gene identified herein.

Determining the presence of a particular variance or plurality of variances in a particular gene in a patient can be performed in a variety of ways. In preferred embodiments, the detection of the presence or absence of at least one variance involves amplifying a segment of nucleic acid including at least one of the at least one variances. Preferably a segment of nucleic acid to be amplified is 500 nucleotides or less in length, more preferably 100 nucleotides or less, and most preferably 45 nucleotides or less. Also, preferably the amplified segment or segments includes a plurality of variances, or a plurality of segments of a gene or of a plurality of genes.

In another aspect determining the presence of a set of variances in a specific gene may entail a haplotyping test that requires allele-specific amplification of a large DNA segment of no greater than 20,000 nucleotides, preferably no greater than 10,000 nucleotides and more preferably no greater than 5,000 nucleotides. Alternatively one allele may be enriched by methods other than amplification prior to determining genotypes at specific variant positions on the enriched allele as a way of determining haplotypes. Preferably the determination of the presence or absence of a variance involves determining the sequence of the variance site or sites by methods such as chain terminating DNA sequencing or minisequencing, or by oligonucleotide hybridization or by mass spectrometry.

The term "genotype" in the context of this invention refers to the particular alleleic form of a gene, which can be defined by the particular nucleotide(s) present in a nucleic acid sequence at a particular site(s).

In preferred embodiments, the detection of the presence or absence of the at least one variance involves contacting a nucleic acid sequence corresponding to one of the genes identified above or a product of such a gene with a probe. The probe is able to distinguish a particular form of the gene or gene product or the presence or a particular variance or variances, e.g., by differential binding or hybridization. Thus, exemplary probes include nucleic acid hybridization probes, peptide nucleic acid probes, nucleotide-containing probes which also contain at least one nucleotide analog, and antibodies, e.g., monoclonal antibodies, and other probes as discussed herein. Those skilled in the art are familiar with the preparation of probes with particular specificities. Those skilled in the art will recognize that a variety of variables can be adjusted to optimize the discrimination between two variant forms of a gene, including changes in salt concentration, temperature, pH and addition of various compounds that affect the differential affinity of GC vs. AT base pairs, such as tetramethyl ammonium chloride. (See Current Protocols in Molecular Biology by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J.G. Seidman, K. Struhl and V. B. Chanda (Editors), John Wiley & Sons.)

In other preferred embodiments, determining the presence or absence of the at least one variance involves sequencing at least one nucleic acid sequence. The sequencing involves sequencing of a portion or portions of a gene and/or portions of a plurality of genes which includes at least one variance site, and may include a plurality of such sites. Preferably, the portion is 500 nucleotides or less in length, more preferably 100 nucleotides or less, and most preferably 45 nucleotides or less in length. Such sequencing can be carried out by various methods recognized by those skilled in the art, including use of dideoxy termination methods (e.g., using dye-labeled dideoxy nucleotides) and the use of mass spectrometric methods. In addition, mass spectrometric methods may be used to determine the nucleotide present at a variance site. In preferred embodiments in which a plurality of variances is determined, the plurality of variances can constitute a haplotype or haplotypes.

The terms "variant form of a gene", "form of a gene", or "allele" refer to one specific form of a gene in a population, the specific form differing from other forms of the same gene in the sequence of at least one, and frequently more than one, variant sites within the sequence of the gene. The sequences at these variant sites that differ between different alleles of the gene are termed "gene sequence variances" or "variances" or "variants". The term "alternative form" refers to an allele that can be distinguished from other alleles by

having distinct variances at at least one, and frequently more than one, variant sites within the gene sequence. Other terms known in the art to be equivalent include mutation and polymorphism, although mutation is often used to refer to an allele associated with a deleterious phenotype. In preferred aspects of this invention, the variances are selected from the group consisting of the variances listed in the variance tables herein or in a patent or patent application referenced and incorporated by reference in this disclosure. In the methods utilizing variance presence or absence, reference to the presence of a variance or variances means particular variances, i.e., particular nucleotides at particular polymorphic sites, rather than just the presence of any variance in the gene.

Variances occur in the human genome at approximately one in every 500 – 1,000 bases within the human genome when two alleles are compared. When multiple alleles from unrelated individuals are compared the frequency of variant sites increases. At most variant sites there are only two alternative nucleotides involving the substitution of one base for another or the insertion/deletion of one or more nucleotides. Within a gene there may be several variant sites. Variant forms of the gene or alternative alleles can be distinguished by the presence of alternative variances at a single variant site, or a combination of several different variances at different sites (haplotypes).

It is estimated that there are 3,300,000,000 bases in the sequence of a single haploid human genome. All human cells except germ cells are normally diploid. Each gene in the genome may span 100-10,000,000 bases of DNA sequence or 100-20,000 bases of mRNA. It is estimated that there are between 60,000 and 120,000 genes in the human genome. The "identification" of genetic variances or variant forms of a gene involves the discovery of variances that are present in a population. The identification of variances is required for development of a diagnostic test to determine whether a patient has a variant form of a gene that is known to be associated with a disease, condition, or predisposition or with the efficacy or safety of the drug. Identification of previously undiscovered genetic variances is distinct from the process of "determining" the status of known variances by a diagnostic test. The present invention provides exemplary variances in genes listed in the gene tables, as well as methods for discovering additional variances in those genes and a comprehensive written description of such additional possible variances. Also described are methods for DNA diagnostic tests to determine the DNA sequence at a particular variant site or sites.

The process of "identifying" or discovering new variances involves comparing the sequence of at least two alleles of a gene, more preferably at least 10 alleles and most preferably at least 50 alleles, (keeping in mind that each somatic cell has two alleles). The analysis of large numbers of individuals to discover variances in the gene sequence between individuals in a population will result in detection of a greater fraction of all the variances in the population. Preferably the process of identifying reveals whether there is a variance within the gene; more preferably identifying reveals the location of the variance within the gene; more preferably identifying provides knowledge of the sequence of the nucleic acid sequence of the variance, and most preferably identifying provides knowledge of the combination of different variances that comprise specific variant forms of the gene or alleles. In identifying new variances it is often useful to screen different population groups based on racial, ethnic, gender, and/or geographic origin because particular variances may differ in frequency between such groups. It may also be useful to screen DNA from individuals with a particular disease or condition of interest because they may have a higher frequency of certain variances than the general population.

The process of determining involves using diagnostic tests for specific variances or variant forms of the gene (or genes) that have been identified within the gene. It will be apparent that such diagnostic tests can only be performed after variances and variant forms of the gene have been identified. Identification of variances can be performed by a variety of methods, alone or in combination, including, for example, DNA sequencing, SSCP, heteroduplex analysis, denaturing gradient gel electrophoresis (DGGE), heteroduplex cleavage (either enzymatic as with T4 Endonuclease 7, or chemical as with osmium tetroxide and hydroxylamine), computational methods (described herein), and other methods described herein as well as others known to those skilled in the art. (See, for example: Cotton, R.G.H., Slowly but surely towards better scanning for mutations, Trends in Genetics 13(2):43-6, 1997, or Current Protocols in Human Genetics by N. C. Dracopoli, J. L. Haines, B. R. Korf, D. T. Moir , C. C. Morton, C. E. Seidman, J.G. Seidman, D. R. Smith and A. Boyle (Editors), John Wiley & Sons.)

In the context of this invention, the term "analyzing a sequence" refers to determining at least some sequence information about the sequence, e.g., determining the nucleotides present at particular sites in the sequence or determining the base sequence of all of a portion of the particular sequence.

In the context of this invention, the term "haplotype" refers to a *cis* arrangement of two or more polymorphic nucleotides, i.e., variances, on a particular chromosome, e.g., in a particular gene. The haplotype preserves the information of the phase of the polymorphic nucleotides – that is, which set of variances were inherited from one parent, and which from the other.

In preferred embodiments of this invention, the frequency of the variance or variant form of the gene in a population is known. Measures of frequency known in the art include "allele frequency", namely the fraction of genes in a population that have one specific variance or set of variances. The allele frequencies for any gene should sum to 1. Another measure of frequency known in the art is the "heterozygote frequency" namely, the fraction of individuals in a population who carry two alleles, or two forms of a particular variance or variant form of a gene, one inherited from each parent. Alternatively, the number of individuals who are homozygous for a particular form of a gene may be a useful measure. The relationship between allele frequency, heterozygote frequency, and homozygote frequency is described for many genes by the Hardy-Weinberg equation, which provides the relationship between allele frequency, heterozygote frequency and homozygote frequency in a freely breeding population at equilibrium. Most human variances are substantially in Hardy-Weinberg equilibrium. In a preferred aspect of this invention, the allele frequency, heterozygote frequency, or homozygote frequency are determined experimentally. Preferably a variance has an allele frequency of at least 0.01, more preferably at least 0.05, still more preferably at least 0.10. However, the allele may have a frequency as low as 0.001 if the associated phenotype is a rare form of toxic reaction to the treatment or drug.

In this regard, "population" refers to a geographically, ethnically, racially, gender, and/or culturally defined group of individuals or a group of individuals with a particular disease or condition or individuals that may be treated with a specific drug. In most cases a population will preferably encompass at least ten thousand, one hundred thousand, one million, ten million, or more individuals, with the larger numbers being more preferable. In a preferred aspect of this invention, the population refers to individuals with a specific disease or condition that may be treated with a specific drug. In an aspect of this invention, the allele frequency, heterozygote frequency, or homozygote frequency of a specific variance or variant form of a gene is known. In preferred embodiments of this invention, the

frequency of one or more variances that may predict response to a treatment is determined in one or more populations using a diagnostic test.

It should be emphasized that it is currently not generally practical to study entire gene sequences in entire populations to establish the association between a specific disease or condition and a specific variance or variant form of the gene. Such studies are commonly performed in controlled clinical trials using a limited number of patients that are considered to be representative of the population with the disease.

In the context of this invention, the term "probe" refers to a molecule which can detectably distinguish between target molecules differing in structure. Detection can be accomplished in a variety of different ways depending on the type of probe used and the type of target molecule. Thus, for example, detection may be based on discrimination of activity levels of the target molecule, but preferably is based on detection of specific binding. Examples of such specific binding include antibody binding and nucleic acid probe hybridization. Thus, for example, probes can include enzyme substrates, antibodies and antibody fragments, and nucleic acid hybridization probes. Thus, in preferred embodiments, the detection of the presence or absence of the at least one variance involves contacting a nucleic acid sequence which includes a variance site with a probe, preferably a nucleic acid probe, where the probe preferentially hybridizes with a form of the nucleic acid sequence containing a complementary base at the variance site as compared to hybridization to a form of the nucleic acid sequence having a non-complementary base at the variance site, where the hybridization is carried out under selective hybridization conditions. Such a nucleic acid hybridization probe may span two or more variance sites. Unless otherwise specified, a nucleic acid probe can include one or more nucleic acid analogs, labels or other substituents or moieties so long as the base-pairing function is retained.

As is generally understood, administration of a particular treatment, e.g., administration of a therapeutic compound or combination of compounds, is chosen depending on the disease or condition which is to be treated. Thus, in certain preferred embodiments, the disease or condition is one for which administration of a treatment is expected to provide a therapeutic benefit; in certain embodiments, the compound is a compound identified herein, e.g., in a drug table such as Tables 7 and 9.

As used herein, the terms "effective" and "effectiveness" includes both pharmacological effectiveness and physiological safety. Pharmacological effectiveness

refers to the ability of the treatment to result in a desired biological effect in the patient. Physiological safety refers to the level of toxicity, or other adverse physiological effects at the cellular, organ and/or organism level (often referred to as side-effects) resulting from administration of the treatment. On the other hand, the term "ineffective" indicates that a treatment does not provide sufficient pharmacological effect to be therapeutically useful, even in the absence of deleterious effects, at least in the total (unstratified) population. (Such a treatment may be effective in a subgroup that can be identified by the presence of one or more sequence variances or alleles.) "Less effective" means that the treatment results in a therapeutically significant lower level of pharmacological effectiveness and/or a therapeutically greater level of adverse physiological effects.

Thus, in connection with the administration of a drug, a drug which is "effective against" a disease or condition indicates that administration in a clinically appropriate manner results in a beneficial effect for at least a statistically significant fraction of patients, such as a improvement of symptoms, a cure, a reduction in disease load, reduction in tumor mass or cell numbers, extension of life, improvement in quality of life, or other effect generally recognized as positive by medical doctors familiar with treating the particular type of disease or condition.

The term "deleterious effects" refers to physical effects in a patient caused by administration of a treatment which are regarded as medically undesirable. Thus, for example, deleterious effects can include a wide spectrum of toxic effects injurious to health such as death of normal cells when only death of diseased cells is desired, nausea, fever, inability to retain food, dehydration, damage to critical organs such as renal tubular necrosis, fatty liver or pulmonary fibrosis, among many others. In this regard, the term "contra-indicated" means that a treatment results in deleterious effects such that a prudent medical doctor treating such a patient would regard the treatment as unsuitable for administration. Major factors in such a determination can include, for example, availability and relative advantages of alternative treatments, consequences of non-treatment, and permanency of deleterious effects of the treatment.

It is recognized that many treatment methods, e.g., administration of certain compounds or combinations of compounds, produces side-effects or other deleterious effects in patients. Such effects can limit or even preclude use of the treatment method in particular patients, or may even result in irreversible injury, dysfunction, or death of the patient. Thus,

in certain embodiments, the variance information is used to select both a first method of treatment and a second method of treatment. Usually the first treatment is a primary treatment which provides a physiological effect directed against the disease or condition or its symptoms. The second method is directed to reducing or eliminating one or more deleterious effects of the first treatment, e.g., to reduce a general toxicity or to reduce a side effect of the primary treatment. Thus, for example, the second method can be used to allow use of a greater dose or duration of the first treatment, or to allow use of the first treatment in patients for whom the first treatment would not be tolerated or would be contra-indicated in the absence of a second method to reduce deleterious effects.

In a related aspect, the invention provides a method for selecting a method of treatment for a patient suffering from a disease or condition by comparing at least one variance in at least one gene in the patient, with a list of variances in the gene or genes which are indicative of the effectiveness of at least one method of treatment. Preferably the comparison involves a plurality of variances or a haplotype indicative of the effectiveness of at least one method of treatment. Also, preferably the list of variances includes a plurality of variances.

Similar to the above aspect, in preferred embodiments the at least one method of treatment involves the administration of a compound effective in at least some patients with a disease or condition; the presence or absence of the at least one variance is indicative that the treatment will be effective in the patient; and/or the presence or absence of the at least one variance is indicative that the treatment will be ineffective or contra-indicated in the patient; and/or the treatment is a first treatment and the presence or absence of the at least one variance is indicative that a second treatment will be beneficial to reduce a deleterious effect of the first treatment; and/or the at least one treatment is a plurality of methods of treatment. For a plurality of treatments, preferably the selecting involves determining whether any of the methods of treatment will be more effective than at least one other of the plurality of methods of treatment. Yet other embodiments are provided as described for the preceding aspect in connection with methods of treatment using administration of a compound; treatment of various diseases, and variances in particular genes.

In the context of variance information in the methods of this invention, the term "list" refers to one or more variances which have been identified for a series or genes of

potential importance in accounting for inter-individual variation in treatment response. Preferably there is a plurality of variances for the gene or genes, preferably a plurality of variances for a particular gene. Preferably the list is recorded in written or electronic form. For example, variances are recorded in Tables 3, 4, 10, and 11 and additional gene variance identification tables herein in a form which allows comparison with other variance information.

In addition to the basic method of treatment, often the mode of administration of a given compound as a treatment for a disease or condition in a patient is significant in determining the course and/or outcome of the treatment for the patient. Thus, the invention also provides a method for selecting a method of administration of a compound to a patient suffering from a disease or condition, by determining the presence or absence of at least one variance in cells of the patient in a gene which is a gene selected from the genes identified in a gene table or list below, where such presence or absence is indicative of an appropriate method of administration of the compound. Preferably, the selection of a method of treatment (a treatment regimen) involves selecting a dosage level or frequency of administration or route of administration of the compound or combinations of those parameters. In preferred embodiments, two or more compounds are to be administered, and the selecting involves selecting a method of administration for one, two, or more than two of the compounds, jointly, concurrently, or separately. As understood by those skilled in the art, such plurality of compounds is often used in combination therapy, and thus may be formulated in a single drug, or may be separate drugs administered concurrently, serially, or separately. Other embodiments are as indicated above for selection of second treatment methods, methods of identifying variances, and methods of treatment as described for aspects above.

In another aspect, the invention provides a method for selecting a patient for administration of a method of treatment for a disease or condition, or of selecting a patient for a method of administration of a treatment, by comparing the presence or absence of at least one variance in a gene as identified above in cells of a patient, with a list of variances in the gene, where the presence or absence of the at least one variance is indicative that the treatment or method of administration will be effective in the patient. If the at least one

variance is present in the patient's cells, then the patient is selected for administration of the treatment.

In preferred embodiments, the disease or the method of treatment is as described in aspects above, specifically including, for example, those described for selecting a method of treatment

In another aspect, the invention provides a method for identifying a subset of patients with enhanced or diminished response or tolerance to a treatment method or a method of administration of a treatment where the treatment is for a disease or condition in the patient. The method involves correlating one or more variances in one or more genes in a plurality of patients with response to a treatment or a method of administration of a treatment. The correlation may be performed by determining the one or more variances in the one or more genes in the plurality of patients and correlating the presence or absence of each of the variances (alone or in various combinations) with the patient's response to treatment. The variances may be previously known to exist or may also be determined in the present method or combinations of prior information and newly determined information may be used. The enhanced or diminished response should be statistically significant, preferably such that $p = 0.10$ or less, more preferably 0.05 or less, and most preferably 0.02 or less. A positive correlation between the presence of one or more variances and an enhanced response to treatment is indicative that the treatment is particularly effective in the group of patients having those variances. A positive correlation of the presence of the one or more variances with a diminished response to the treatment is indicative that the treatment will be less effective in the group of patients having those variances. Such information is useful, for example, for selecting or de-selecting patients for a particular treatment or method of administration of a treatment, or for demonstrating that a group of patients exists for which the treatment or method of treatment would be particularly beneficial or contra-indicated. Such demonstration can be beneficial, for example, for obtaining government regulatory approval for a new drug or a new use of a drug.

In preferred embodiments, the variances are in particular genes, or are particular variances described herein. Also, preferred embodiments include drugs, treatments, variance identification or determination, determination of effectiveness, lists, and/or diseases as described for aspects above or otherwise described herein.

In preferred embodiments, the correlation of patient responses to therapy according to patient genotype is carried out in a clinical trial, e.g., as described herein according to any of the variations described.. Detailed description of methods for associating variances with clinical outcomes using clinical trials are provided below.

As indicated above, in aspects of this invention involving selection of a patient for a treatment, selection of a method or mode of administration of a treatment, and selection of a patient for a treatment or a method of treatment, the selection may be positive selection or negative selection. Thus, the methods can include eliminating a treatment for a patient, eliminating a method or mode of administration of a treatment to a patient, or elimination of a patient for a treatment or method of treatment.

Also, in methods involving identification and/or comparison of variances present in a gene of a patient, the methods can involve such identification or comparison for a plurality of genes. Preferably, the genes are functionally related to the same disease or condition, or to the aspect of disease pathophysiology that is being subjected to pharmacological manipulation by the treatment (e.g. a drug), or to the activation or inactivation of the drug, and more preferably the genes are involved in the same biochemical process or pathway.

In another aspect, the invention provides a method for identifying the forms of a gene in an individual, where the gene is one specified as for aspects above, by determining the presence or absence of at least one variance in the gene. In preferred embodiments, the at least one variance includes at least one variance selected from the group of variances identified in variance tables herein. Preferably, the presence or absence of the at least one variance is indicative of the effectiveness of a therapeutic treatment in a patient suffering from a disease or condition and having cells containing the at least one variance.

The presence or absence of the variances can be determined in any of a variety of ways as recognized by those skilled in the art. For example, the nucleotide sequence of at least one nucleic acid sequence which includes at least one variance site (or a complementary sequence) can be determined, such as by chain termination methods, hybridization methods or by mass spectrometric methods. Likewise, in preferred embodiments, the determining involves contacting a nucleic acid sequence or a gene product of one of one of the genes with a probe which specifically identifies the presence or absence

of a form of the gene. For example, a probe, e.g., a nucleic acid probe, can be used which specifically binds, e.g., hybridizes, to a nucleic acid sequence corresponding to a portion of the gene and which includes at least one variance site under selective binding conditions. As described for other aspects, determining the presence or absence of at least two variances can constitute determining a haplotype or haplotypes.

Other preferred embodiments involve variances related to types of treatment, drug responses, diseases, nucleic acid sequences, and other items related to variances and variance determination as described for aspects above.

In yet another aspect, the invention provides a pharmaceutical composition which includes a compound which has a differential effect in patients having at least one copy, or alternatively, two copies of a form of a gene as identified for aspects above and a pharmaceutically acceptable carrier, excipient, or diluent. The composition is adapted to be preferentially effective to treat a patient with cells containing the one, two, or more copies of the form of the gene.

In preferred embodiments of aspects involving pharmaceutical compositions, active compounds, or drugs, the material is subject to a regulatory limitation or restriction on approved uses or indications, e.g., by the U.S. Food and Drug Administration (FDA), limiting approved use of the composition to patients having at least one copy of the particular form of the gene which contains at least one variance. Alternatively, the composition is subject to a regulatory limitation or restriction on approved uses indicating that the composition is not approved for use or should not be used in patients having at least one copy of a form of the gene including at least one variance. Also in preferred embodiments, the composition is packaged, and the packaging includes a label or insert indicating or suggesting beneficial therapeutic approved use of the composition in patients having one or two copies of a form of the gene including at least one variance. Alternatively, the label or insert limits approved use of the composition to patients having zero or one or two copies of a form of the gene including at least one variance. The latter embodiment would be likely where the presence of the at least one variance in one or two copies in cells of a patient means that the composition would be ineffective or deleterious to the patient. Also in preferred embodiments, the composition is indicated for use in treatment of a disease or condition which is one of those identified for aspects above. Also in

preferred embodiments, the at least one variance includes at least one variance from those identified herein.

The term "packaged" means that the drug, compound, or composition is prepared in a manner suitable for distribution or shipping with a box, vial, pouch, bubble pack, or other protective container, which may also be used in combination. The packaging may have printing on it and/or printed material may be included in the packaging.

In preferred embodiments, the drug is selected from the drug classes or specific exemplary drugs identified in an example, in a table or list herein, and is subject to a regulatory limitation or suggestion or warning as described above that limits or suggests limiting approved use to patients having specific variances or variant forms of a gene identified in Examples or in a gene list provided below in order to achieve maximal benefit and avoid toxicity or other deleterious effect.

A pharmaceutical composition can be adapted to be preferentially effective in a variety of ways. In some cases, an active compound is selected which was not previously known to be differentially active, or which was not previously recognized as a potential therapeutic compound. In some cases, the concentration of an active compound which has differential activity can be adjusted such that the composition is appropriate for administration to a patient with the specified variances. For example, the presence of a specified variance may allow or require the administration of a much larger dose, which would not be practical with a previously utilized composition. Conversely, a patient may require a much lower dose, such that administration of such a dose with a prior composition would be impractical or inaccurate. Thus, the composition may be prepared in a higher or lower unit dose form, or prepared in a higher or lower concentration of the active compound or compounds. In yet other cases, the composition can include additional compounds needed to enable administration of a particular active compound in a patient with the specified variances, which was not in previous compositions, e.g., because the majority of patients did not require or benefit from the added component.

The term "differential" or "differentially" generally refers to a statistically significant different level in the specified property or effect. Perferably, the difference is also functionally significant. Thus, "differential binding or hybridization" is sufficient difference in binding or hybridization to allow discrimination using an appropriate detection technique. Likewise, "differential effect" or "differentially active" in connection with a therapeutic

treatment or drug refers to a difference in the level of the effect or activity which is distinguishable using relevant parameters and techniques for the effect or activity being considered. Preferably the difference in effect or activity is also sufficient to be clinically significant, such that a corresponding difference in the course of treatment or treatment outcome would be expected, at least on a probabilistic basis.

Also usefully provided in the present invention are probes which specifically recognize a nucleic acid sequence corresponding to a variance or variances in a gene or a product expressed from the gene, and are able to distinguish a variant form of the sequence or gene or gene product from one or more other variant forms of that sequence, gene, or gene product under selective conditions. Those skilled in the art recognize and understand the identification or determination of selective conditions for particular probes or types of probes. An exemplary type of probe is a nucleic acid hybridization probe, which will selectively bind under selective binding conditions to a nucleic acid sequence or a gene product corresponding to one or the genes identified for aspects above. Another type of probe is a peptide or protein, e.g., an antibody or antibody fragment which specifically or preferentially binds to a polypeptide expressed from a particular form of a gene as characterized by the presence or absence of at least one variance. Thus, in another aspect, the invention concerns such probes. In the context of this invention, a "probe" is a molecule, commonly a nucleic acid, though also potentially a protein, carbohydrate, polymer, or small molecule, that is capable of binding to one variance or variant form of the gene or gene product to a greater extent than to a form of the gene having a different base at one or more variance sites, such that the presence of the variance or variant form of the gene can be determined. Preferably the probe distinguishes at least one variance identified in Examples, tables or lists below. Preferably the probe also has specificity for the particular gene or gene product, at least to an extent such that binding to other genes or gene products does not prevent use of the assay to identify the presence or absence of the particular variance or variances of interest.

In preferred embodiments,the probe is an antibody or antibody fragment. Such antibodies may be polyclonal or monoclonal antibodies, and can be prepared by methods well-known in the art. In preferred embodiments, the probe is a nucleic acid probe at least 15, preferably at least 17 nucleotides in length, more preferably at least 20 or 22 or 25,

preferably 500 or fewer nucleotides in length, more preferably 200 or 100 or fewer, still more preferably 50 or fewer, and most preferably 30 or fewer. In preferred embodiments, the probe has a length in a range between from any one of the above lengths to any other of the above lengths (including endpoints). The probe specifically hybridizes under selective hybridization conditions to a nucleic acid sequence corresponding to a portion of one of the genes identified in connection with above aspects. The nucleic acid sequence includes at least one and preferably two or more variance sites. Also in preferred embodiments, the probe has a detectable label, preferably a fluorescent label. A variety of other detectable labels are known to those skilled in the art. Such a nucleic acid probe can also include one or more nucleic acid analogs.

In preferred embodiments, the probe is an antibody or antibody fragment which specifically binds to a gene product expressed from a form of one of the above genes, where the form of the gene has at least one specific variance with a particular base at the variance site, and preferably a plurality of such variances.

In connection with nucleic acid probe hybridization, the term "specifically hybridizes" indicates that the probe hybridizes to a sufficiently greater degree to the target sequence than to a sequence having a mismatched base at at least one variance site to allow distinguishing such hybridization. The term "specifically hybridizes" thus means that the probe hybridizes to the target sequence, and not to non-target sequences, at a level which allows ready identification of probe/target sequence hybridization under selective hybridization conditions. Thus, "selective hybridization conditions" refer to conditions which allow such differential binding. Similarly, the terms "specifically binds" and "selective binding conditions" refer to such differential binding of any type of probe, e.g., antibody probes, and to the conditions which allow such differential binding. Typically hybridization reactions to determine the status of variant sites in patient samples are carried out with two different probes, one specific for each of the (usually two) possible variant nucleotides. The complementary information derived from the two separate hybridization reactions is useful in corroborating the results.

Likewise, the invention provides an isolated, purified or enriched nucleic acid sequence of 15 to 500 nucleotides in length, preferably 15 to 100 nucleotides in length, more preferably 15 to 50 nucleotides in length, and most preferably 15 to 30 nucleotides in length,

which has a sequence which corresponds to a portion of one of the genes identified for aspects above. Preferably the lower limit for the preceding ranges is 17, 20, 22, or 25 nucleotides in length. In other embodiments, the nucleic acid sequence is 30 to 300 nucleotides in length, or 45 to 200 nucleotides in length, or 45 to 100 nucleotides in length. The nucleic acid sequence includes at least one variance site. Such sequences can, for example, be amplification products of a sequence which spans or includes a variance site in a gene identified herein. Likewise, such a sequence can be a primer which is able to bind to or extend through a variance site in such a gene. Yet another example is a nucleic acid hybridization probe comprised of such a sequence. In such probes, primers, and amplification products, the nucleotide sequence can contain a sequence or site corresponding to a variance site or sites, for example, a variance site identified herein. Preferably the presence or absence of a particular variant form in the heterozygous or homozygous state is indicative of the effectiveness of a method of treatment in a patient.

Typically primers are utilized in pairs. Primers can be designed or selected by methods well-known to those skilled in the art based on nucleotide sequences corresponding to at least a portion or a gene identified herein. The primer or primers hybridizes to or allows amplification (e.g., using the polymerase chain reaction) through a nucleic acid sequence containing at least one sequence variance. Preferably such primers hybridize to a sequence not more than 300 nucleotides, more preferably not more than 200 nucleotides, still more preferably not more than 100 nucleotides, and most preferably not more than 50 nucleotides away from a variance site which is to be analyzed. Preferably, a primer is 100 nucleotides or fewer in length, more preferably 50 nucleotides or fewer, still more preferable 30 nucleotides or fewer, and most preferably 20 or fewer nucleotides in length.

In reference to nucleic acid sequences which "correspond" to a gene, the term "correspond" refers to a nucleotide sequence relationship, such that the nucleotide sequence has a nucleotide sequence which is the same as the reference gene or an indicated portion thereof, or has a nucleotide sequence which is exactly complementary in normal Watson-Crick base pairing, or is an RNA equivalent of such a sequence, e.g., a mRNA, or is a cDNA derived from an mRNA of the gene.

In a related aspect, the invention provides a kit containing at least one probe or at least one primer or both (e.g., as described above) corresponding to a gene or genes of this

invention. The kit is preferably adapted and configured to be suitable for identification of the presence or absence of a particular variance or variances, which can include or consist of sequence a nucleic acid sequence corresponding to a portion of a gene. The kit may also contain a plurality of either or both of such probes and/or primers, e.g., 2, 3, 4, 5, 6, or more of such probes and/or primers. Preferably the plurality of probes and/or primers are adapted to provide detection of a plurality of different sequence variances in a gene or plurality of genes, e.g., in 2, 3, 4, 5, or more genes or to sequence a nucleic acid sequence including at least one variance site in a gene or genes. Preferably one or more of the variance or variances to be detected are correlated with variability in a treatment response or tolerance, and are preferably indicative of an effective response to a treatment. In preferred embodiments, the kit contains components (e.g., probes and/or primers) adapted or useful for detection of a plurality of variances (which may be in one or more genes) indicative of the effectiveness of at least one treatment, preferably of a plurality of different treatments for a particular disease or condition. It may also be desirable to provide a kit containing components adapted or useful to allow detection of a plurality of variances indicative of the effectiveness of a treatment or treatment against a plurality of diseases. The kit may also optionally contain other components, preferably other components adapted for identifying the presence of a particular variance or variances. Such additional components can, for example, independently include a buffer or buffers, e.g., amplification buffers and hybridization buffers, which may be in liquid or dry form, a DNA polymerase, e.g., a polymerase suitable for carrying out PCR, and deoxy nucleotide triphosphases (dNTPs). Preferably a probe includes a detectable label, e.g., a fluorescent label, enzyme label, light scattering label, or other label. Preferably the kit includes a nucleic acid or polypeptide array. The array may, for example, include a plurality of different antibodies, a plurality of different nucleic acid sequences. Sites in the array can allow capture and/or detection of nucleic acid sequences or gene products corresponding to different variances in one or more different genes. Preferably the array is arranged to provide variance detection for a plurality of variances in one or more genes which correlate with the effectiveness of one or more treatments of one or more diseases.

The kit may also optionally contain instructions for use, which can include a listing of the variances correlating with a particular treatment or treatments for a disease of diseases.

Preferably the kit components are selected to allow detection of a variance described herein, and/or detection of a variance indicative of a treatment,e.g., administration of a drug, pointed out herein.

Additional configurations for kits of this invention will be apparent to those skilled in the art.

In another aspect, the invention provides a method for determining a genotype of an individual in relation to one or more variances in one or more of the genes identified in above aspects by using mass spectrometric determination of a nucleic acid sequence which is a portion of a gene identified for other aspects of this invention or a complementary sequence. Such mass spectrometric methods are known to those skilled in the art. In preferred embodiments, the method involves determining the presence or absence of a variance in a gene; determining the nucleotide sequence of the nucleic acid sequence; the nucleotide sequence is 100 nucleotides or less in length, preferably 50 or less, more preferably 30 or less, and still more preferably 20 nucleotides or less. In general, such a nucleotide sequence includes at least one variance site, preferably a variance site which is informative with respect to the expected response of a patient to a treatment as described for above aspects.

As indicated above, many therapeutic compounds or combinations of compounds or pharmaceutical compositions show variable efficacy and/or safety in various patients in whom the compound or compounds is administered. Thus, it is beneficial to identify variances in relevant genes, e.g., genes related to the action or toxicity of the compound or compounds. Thus, in a further aspect, the invention provides a method for determining whether a compound has a differential effect due to the presence or absence of at least one variance in a gene or a variant form of a gene, where the gene is a gene identified for aspects above.

The method involves identifying a first patient or set of patients suffering from a disease or condition whose response to a treatment differs from the response (to the same treatment) of a second patient or set of patients suffering from the same disease or condition, and then determining whether the frequency of at least one variance in at least one gene differs in frequency between the first patient or set of patients and the second patient or set

of patients. A correlation between the presence or absence of the variance or variances and the response of the patient or patients to the treatment indicates that the variance provides information about variable patient response. In general, the method will involve identifying at least one variance in at least one gene. An alternative approach is to identify a first patient or set of patients suffering from a disease or condition and having a particular genotype, haplotype or combination of genotypes or haplotypes, and a second patient or set of patients suffering from the same disease or condition that have a genotype or haplotype or sets of genotypes or haplotypes that differ in a specific way from those of the first set of patients. Subsequently the extent and magnitude of clinical response can be compared between the first patient or set of patients and the second patient or set of patients. A correlation between the presence or absence of a variance or variances or haplotypes and the response of the patient or patients to the treatment indicates that the variance provides information about variable patient response and is useful for the present invention.

The method can utilize a variety of different informative comparisons to identify correlations. For example a plurality of pairwise comparisons of treatment response and the presence or absence of at least one variance can be performed for a plurality of patients. Likewise, the method can involve comparing the response of at least one patient homozygous for at least one variance with at least one patient homozygous for the alternative form of that variance or variances. The method can also involve comparing the response of at least one patient heterozygous for at least one variance with the response of at least one patient homozygous for the at least one variance. Preferably the heterozygous patient response is compared to both alternative homozygous forms, or the response of heterozygous patients is grouped with the response of one class of homozygous patients and said group is compared to the response of the alternative homozygous group.

Such methods can utilize either retrospective or prospective information concerning treatment response variability. Thus, in a preferred embodiment, it is previously known that patient response to the method of treatment is variable.

Also in preferred embodiments, the disease or condition is as for other aspects of this invention; for example, the treatment involves administration of a compound or pharmaceutical composition.

In preferred embodiments, the method involves a clinical trial, e.g., as described herein. Such a trial can be arranged, for example, in any of the ways described herein, e.g., in the Detailed Description.

The present invention also provides methods of treatment of a disease or condition. Such methods combine identification of the presence or absence of particular variances with the administration of a compound; identification of the presence of particular variances with selection of a method of treatment and administration of the treatment; and identification of the presence or absence of particular variances with elimination of a method of treatment based on the variance information indicating that the treatment is likely to be ineffective or contra-indicated, and thus selecting and administering an alternative treatment effective against the disease or condition. Thus, preferred embodiments of these methods incorporate preferred embodiments of such methods as described for such sub-aspects.

As used herein, a "gene" is a sequence of DNA present in a cell that directs the expression of a "biologically active" molecule or "gene product", most commonly by transcription to produce RNA and translation to produce protein. The "gene product' is most commonly a RNA molecule or protein or a RNA or protein that is subsequently modified by reacting with, or combining with, other constituents of the cell. Such modifications may include, without limitation, modification of proteins to form glycoproteins, lipoproteins, and phosphoproteins, or other modifications known in the art. RNA may be modified without limitation by complexing with proteins, polyadenylation, splicing, capping or export from the nucleus. The term "gene product" refers to any product directly resulting from transcription of a gene. In particular this includes partial, precursor, and mature transcription products (i.e, pre-mRNA and mRNA), and translation products with or without further processing including, without limitation, lipidation, phosphorylation, glycosylation, or combinations of such processing

The term "gene involved in the origin or pathogenesis of a disease or condition" refers to a gene that harbors mutations that contribute to the cause of disease, or variances that affect the progression of the disease or expression of specific characteristic of the disease. The term also applies to genes involved in the synthesis, accumulation, or elimination of products that are involved in the origin or pathogenesis of a disease or

condition including, without limitation, proteins, lipids, carbohydrates, hormones, or small molecules.

The term "gene involved in the action of a drug" refers to any gene whose gene product affects the efficacy or safety of the drug or affects the disease process being treated by the drug, and includes, without limitation, genes that encode gene products that are targets for drug action, gene products that are involved in the metabolism, activation or degradation of the drug, gene products that are involved in the bioavailability or elimination of the drug to the target, gene products that affect biological pathways that, in turn, affect the action of the drug such as the synthesis or degradation of competitive substrates or allosteric effectors or rate limiting reaction, or, alternatively, gene products that affect the pathophysiology of the disease process. (Particular variances in the latter category of genes may be associated with patient groups in whom disease etiology is more or less susceptible to amelioration by the drug. For example, there are several pathophysiological mechanisms in hypertension, and depending on the dominant mechanism in a given patient, that patient may be more or less likely than the average hypertensive patient to respond to a drug that primarily targets one pathophysiological mechanism. The relative importance of different pathophysiological mechanisms in individual patients is likely to be affected by variances in genes associated with the disease pathophysiology. The "action" of a drug refers to its effect on biological products within the body. The action of a drug also refers to its effects on the signs or symptoms of a disease or condition, or effects of the drug that are unrelated to the disease or condition leading to unanticipated effects on other processes. Such unanticipated processes often lead to adverse events or toxic effects. The terms "adverse event" or "toxic" event" are known in the art and include, without limitation, those listed in the FDA reference system for adverse events.

In accordance with the aspects above and the Detailed Description below, there is also described for this invention an approach or method for developing drugs that are explicitly indicated for, and/or for which approved use is restricted to individuals in the population with specific variances or combinations of variances, as determined by diagnostic tests for variances or variant forms of certain genes involved in the disease or condition or involved in the action of the drug. Such drugs may provide more effective treatment for a disease or condition in a population identified or characterized with the use of a diagnostic test for a specific variance or variant form of the gene if the gene is involved in the action of

the drug or in determining a characteristic of the disease or condition. Such drugs may be developed using the diagnostic tests for specific variances or variant forms of a gene to determine the inclusion of patients in a clinical trial.

Thus, the invention also provides a method for producing a pharmaceutical composition by identifying a compound which has differential activity against a disease or condition in patients having at least one variance in a gene, compounding the pharmaceutical composition by combining the compound with a pharmaceutically acceptable carrier, excipient, or diluent such that the composition is preferentially effective in patients who have at least one copy of the variance or variances. In some cases, the patient has two copies of the variance or variances. In preferred embodiments, the disease or condition, gene or genes, variances, methods of administration, or method of determining the presence or absence of variances is as described for other aspects of this invention.

Similarly, the invention provides a method for producing a pharmaceutical agent by identifying a compound which has differential activity against a disease or condition in patients having at least one copy of a form of a gene having at least one variance and synthesizing the compound in an amount sufficient to provide a pharmaceutical effect in a patient suffering from the disease or condition. The compound can be identified by conventional screening methods and its activity confirmed. For example, compound libraries can be screened to identify compounds which differentially bind to products of variant forms of a particular gene product, or which differentially affect expression of variant forms of the particular gene, or which differentially affect the activity of a product expressed from such gene. Preferred embodiments are as for the preceding aspect.

In another aspect, the invention provides a method of treating a disease or condition in a patient by selecting a patient whose cells have an allele of a gene selected from the genes listed herein, preferably in Tables 2, 6, 8, or 10. The allele contains at least one variance correlated with more effective response to a treatment of the disease or condition, or tolerance of a treatment, e.g., a treatment with a drug or a drug of a class indicated herein.

Preferably the allele contains a variance as shown in 2, 4, 6, or 8 or other variance table herein. Also preferably, the altering involves administering to the patient a compound preferentially active on at least one but less than all alleles of the gene.
Preferred embodiments include those as described above for other aspects of treating a disease or condition.

In a further aspect, the invention provides a method for determining a method of treatment effective to treat a disease or condition by altering the level of activity of a product of an allele of a gene selected from the genes listed in Table 2, 6, or 8, and determining whether that alteration provides a differential effect related to reducing or alleviating a disease or condition as compared to at least one alternative allele or an alteration in toxicity or tolerance of the treatment by a patient or patients. The presence of such a differential effect indicates that altering that level of activity provides at least part of an effective treatment for the disease or condition.

Preferably the determining is carried out in a clinical trial, e.g., as described above and/or in the Detailed Description below.

In still another aspect, the invention provides a method for evaluating differential efficacy of or tolerance to a treatment in a subset of patients who have a particular variance or variances in at least one gene by utilizing a clinical trial. In preferred embodiments, the clinical trial is a Phase I, II, III, or IV trial. Preferred embodiments include the stratifications and/or analyses as described below in the Detailed Description.

In yet another aspect, the invention provides a method for identifying at least one variance in at least one gene using computer-based sequence analysis or variance scanning as known to those skilled in the art.

Preferably the at least one gene is a plurality of genes, preferably at least 10, 20, 50, 100, 200, 500, 1000, 5000, 10,000, or even more. Preferably sequence and/or variance information on the plurality of genes is acumulated in one database or a set of commonly accessible databases within a single local computer network or on a single computer.

In yet another aspect, the invention provides experimental methods for finding additional variances in any of the genes provided in the table of Table 2, 6, or 8. In addition to the sequence analysis method, a number of experimental methods can also beneficially be used to identify variances. Thus the invention provides methods for producing cDNA (e.g., example 13) or genomic DNA and detecting additional variances in the genes provided in Table 2, 6, or 8 using the single strand conformation polymorphism (SSCP) method (Example 14), the T4 Endonuclease VII method (Example 15) or DNA sequencing (Example 16) or other methods pointed out below. The application of these methods to the identified genes will provide identification of additional variances that can affect inter-

individual variation in drug or other treatment response. One skilled in the art will recognize that many methods for experimental variance detection have been described (in addition to the exemplary methods of examples 14, 15 and 16) which can be utilized. These additional methods include chemical cleavage of mismatches (see, e.g., Ellis TP, et al., Chemical cleavage of mismatch: a new look at an established method. *Human Mutation* 11(5):345-53, 1998), denaturing gradient gel electrophoresis (see, e.g., Van Orsouw NJ, et al., Design and application of 2-D DGGE-based gene mutational scanning tests. *Genet Anal.* 14(5-6):205-13, 1999) and heteroduplex analysis (see, e.g., Ganguly A, et al., Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. *Proc Natl Acad Sci USA.* 90 (21):10325-9, 1993).

In embodiments any of the above methods involving determination of the presence or absence of a particular variance or variances, the method preferably involves determining the presence or absence using a cell sample from an individual or individuals. Thus, the methods can also involve obtaining a cell sample from an individual. The cell sample can be any of a variety of different cells, e.g., blood cells skin cells, muscle cells, normal cells, or cancer cells.

By "comprising" is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram showing the relationships of enzymes involved in 5-FU metabolism and inhibition of thymidylate formation. Enzymes: 1. uridine phosphorylase; 2. thymidine phosphorylase; 3. orotate phosphoribosyl transferase; 4. thymidine kinase; 5. uridine kinase; 6. ribonucletide reductase; 7. thymidylate synthase; 8. dCMP deaminase; 9. nucleoside monophosphate kinase; 10. nucleoside diphosphate kinase; 11. nucleoside diphosphatase or cytidylate kinase; 12: thymine phosphorylase. FH2 = dihydrofolate, FH4 = tetrahydrofolate. The Figure is adapted from Goodman & Gilman's The Pharmacological Basis of Therapeutics, ninth edition, McGraw Hill, 1996, p. 1249.

Figure 2 is a diagram showing the relationship of enzymes related to folate metabolism and formation of 5,10-methylenetetrahydrofolate. Enzymes: 1. Forminino-tetrahydrofolate cyclodeaminase; 2. methenyltetrahydrofolate synthetase; 3. methenyltetra-hydrofolate cyclohydrolase; 4. formyltetrahydrofolate synthetase; 5. formyltetrahydrofolate hydrolase; 6. formyltetrahydrofolate dehydrogenase; 7. methyleneltetrahydrofolate dehydrogenase; 8. methyleneltetrahydrofolate reductase (MTHFR); 9. homocysteine methyltransferase (also called methionine synthetase); 10. serine transhydroxymethylase; 11. glycine cleavage system; 12. thymidylate synthase; 13. dihydrofolate reductase. Abbreviations: THF = tetrahydrofolate; DHF = dihydrofolate. Note that THF appears twice (i.e. the product of step 6 is also substrate for enzymes 10 and 11. Step 12 also appears in Figure 1, above. This Figure is adapted from Mathews & van Holde, Biochemistry, The Benjamin/Cummings Publishing Co., Redwood City CA, 1990, page 697.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Tables 10 and 11 will first be briefly described.

Table 10 lists DNA sequence variances in genes relevant to the methods described in the present invention. These variances were identified by the inventors in studies of selected genes, and are provided here as useful for the methods of the present invention. The variances in Table 10 were discovered by one or more of the methods described below in the Detailed Description or Examples. Table 10 has eight columns. Column 1, the "Name" column, contains the Human Genome Organization (HUGO)

identifier for the gene. Column 2, the "GID" column provides the GenBank accession number of a genomic, cDNA, or partial sequence of a particular gene. Column 3, the "OMIM_ID" column contains the record number corresponding to the Online Mendelian Inheritance in Man database for the gene provided in columns 1 and 2. This record number can be entered at the world wide web site http://www3.ncbi.nlm.nih.gov/Omim/searchomim.html to search the OMIM record on the gene. Column 4, the VGX_Symbol column, provides an internal identifier for the gene. Column 5, the "Description" column provides a descriptive name for the gene, when available. Column 6, the "Variance_Start" column provides the nucleotide location of a variance with respect to the first listed nucleotide in the GenBank accession number provided in column 2. That is, the first nucleotide of the GenBank accession is counted as nucleotide 1 and the variant nucleotide is numbered accordingly. Column 7, the "variance" column provides the nucleotide location of a variance with respect to an ATG codon believed to be the authentic ATG start codon of the gene, where the A of ATG is numbered as one (1) and the immediately preceding nucleotide is numbered as minus one (-1). This reading frame is important because it allows the potential consequence of the variant nucleotide to be interpreted in the context of the gene anatomy (5' untranslated region, protein coding sequence, 3' untranslated region). Column 7 also provides the identity of the two variant nucleotides at the indicated position. Column 8, the "CDS_Context" column indicates whether the variance is in a coding region but silent (S); in a coding region and results in an amino acid change (e.g., R347C, where the letters are one letter amino acid abbreviations and the number is the amino acid residue in the encoded amino acid sequence which is changed); in a sequence 5' to the coding region (5); or in a sequence 3' to the coding region (3). As indicated above, interpreting the location of the variance in the gene depends on the correct assignment of the initial ATG of the encoded protein (the translation start site). It should be recognized that assignment of the correct ATG may occasionally be incorrect in GenBank, but that one skilled in the art will know how to carry out experiments to definitively identify the correct translation initiation codon (which is not always an ATG). In the event of any potential question concerning the proper identification of a gene or part of a gene, due for example, to an error in recording an identifier or the absence of one or more of the identifiers, the priority for use to resolve the ambiguity is GenBank accession number, OMIM identification number, HUGO identifier, common name identifier.

If a haplotype for any of the genes listed in this table has been identified, a series of nucleotides (A, C, G, T) are listed separated by commas and to the left of each listing is the associated nucleotide location also separated by commas in brackets. For

example, if the haplotype listing is T,G,C,A [12, 245, 385, 612] there is a T at position 12, a G at position 246, a C at position 385, and an A at position 612. Below this list will occur the identified variance start, variance, and CDS context for the identified single nucleotide polymorphisms as described above.

Table 11 lists additional DNA sequence variances (in addition to those in Table 10) in genes relevant to the methods of the present invention (i.e. selected genes from Table 1). These variances were identified by various research groups and published in the scientific literature over the past 20 years. The inventors realized that these variances may be useful for understanding interpatient variation in response to treatment of the diseases listed herein, and more generally useful for the methods of the present invention. The columns of Table 11 are similar to those of Table 10, and therefore the descriptions of the rows and columns in Table 10 (above) pertain to Table 11, as do the other remarks.

The present invention is generally described below in connection with cancer chemotherapy. However, the described approach and techniques are applicable to a variety of other treatments and to genes associated with the efficacy and safety of such other treatments, for example, genes function in the pathways identified below, along with the specific genes listed. The present invention identifies a number of genes in certain treatment-related pathways, and further identifies a number of genetic sequence variances in those genes. The present description further describes how to identify variances which correlate with variable treatment efficacy and further how to identify additional variances in the identified genes and how to determine the treatment response correlation of those additional variances.

Chemotherapy of cancer currently involves use of highly toxic drugs with narrow therapeutic indices. Although progress has been made in the chemotherapeutic treatment of selected malignancies, most adult solid cancers remain highly refractory to treatment. Nonetheless, chemotherapy is the standard of care for most disseminated solid cancers. Chemotherapy often results in a significant fraction of treated patients suffering unpleasant or life-threatening side effects while receiving little or no clinical benefit; other patients may suffer few side effects and/or have complete remission or even cure. Any test that could predict response to chemotherapy, even partially, would allow more selective use of toxic drugs, and could thereby significantly improve efficacy of oncologic drug use, with the potential to both reduce side effects and increase the fraction of responders. Chemotherapy is also expensive, not just because the drugs are often costly, but also because administering highly toxic drugs requires

close monitoring by carefully trained personnel, and because hospitalization is often required for treatment of (or monitoring for) toxic drug reactions. Information that would allow patients to be divided into likely responder vs. non-responder (or likely side effect) groups, with only the former to receive treatment, would therefore also have a significant impact on the economics of cancer drug use.

Predicting Response to Chemotherapy

Several methods for predicting response to chemotherapy in individual patients have been investigated over the years, ranging from the use of biochemical markers to testing drugs on a patient's cultured tumor cells. None of these methods has proven sufficiently informative and practical to gain wide acceptance. However, there are some specific examples of tests useful for predicting toxicity. For example, a diagnostic test to predict side effects associated with the antineoplastic drugs 6-mercaptopurine, 6-thioguanine and azathioprine has begun to gain wide acceptance, particularly among pediatric oncologists. Severe toxicity of thiopurine drugs is associated with deficiency of the enzyme thiopurine methyltransferase (TPMT). Currently most TPMT testing is done using an enzyme assay, however the TPMT gene has been cloned and mutations associated with low TPMT levels have been identified; genetic testing is beginning to supplant enzyme assays because genetic tests are more easily standardized and economical.

While there are no good tests that predict positive chemotherapeutic response, there is demonstrated utility to measuring estrogen and progesterone receptor levels in cancer tissue before selecting therapy directed at modulating hormonal state. Measuring genetic variation in proteins that mediate the effects, course, outcome, and/or development of adverse events in those patients potentially receiving chemotherapy drugs is, in some respects, analogous to measuring ER and PR levels, which mediate the effects of hormones.

I. Outline: Identification of interpatient variation in response; identification of genes and variances relevant to drug action; development of diagnostic tests; and use of variance status to determine treatment

Human therapeutic development follows a course from discovery and analysis in a laboratory (preclinical development) to testing the candidate therapeutic intervention in human subjects (clinical development). The preclinical development of candidate therapeutic interventions for use in the treatment of human disease, disorders, or conditions begins at the discovery stage whereby a candidate therapy is tested *in vitro* to achieve a desired biochemical alteration of a biochemical or physiological event. If successful, the

candidate is generally tested in animals to determine toxicity, adsorption, distribution, and metabolism within a living species. Occasionally, there are available animal models that mimic human diseases, disorders, and conditions in which testing the candidate therapeutic intervention can provide supportive data to warrant proceeding to test the agent or compound in humans. When an agent or compound enters first in human studies, it is recognized that the prediction of whether the agent or product's preclinical success will be mimicked in humans is imperfect. Both safety and efficacy data will generally have to ultimately be determined in humans. Therefore, given economic constraints, and considering the complexities of human clinical trials, any technical advance to assist those skilled in the art of drug development will be welcomed. Advances can be implemented by aiding identification of genetic markers associated with interpatient variation in response during preclinical development (thereby allowing development of non-allele selective agents), or by identification or optimization of clinical trial design parameters in order to achieve successful development of therapeutic products at any stage of clinical development, or by identifying variables that will allow safe and efficacious use of a marketed product. Such advances will provide benefits in the form of therapeutic alternatives to those patients in need of medical care.

As indicated in the Summary above, certain aspects of the present invention typically involve the following process, which need not occur separately or in the order stated. Not all of these described processes must be present in a particular method, or need be performed by a single entity or organization or person. Additionally, if certain of the information is available from other sources, that information can be utilized in the present invention. The processes are as follows: a) variability between patients in the response to a particular treatment is observed; b) at least a portion of the variable response is correlated with the presence or absence of at least one variance in at least one gene; c) an analytical or diagnostic test is provided to determine the presence or absence of the at least one variance in individual patients; d) the presence or absence of the variance or variances is used to select a patient for a treatment or to select a treatment for a patient, or the variance information is used in other methods described herein.

A. Identification of Interpatient Variability in Response to a Treatment

Interpatient variability is the rule, not the exception, in clinical therapeutics. One of the best sources of information on interpatient variability is the nurses and physicians supervising the clinical trial who accumulate a body of first hand observations of physiological responses to the drug in different normal subjects or patients. Evidence of interpatient variation in response can also be measured statistically, and may be best

described by statistical measures that examine magnitude of response (beneficial or adverse) across a large number of subjects.

In accord with the other portions of this description, the present invention concerns DNA sequence variances that can affect one or more of:

i. The susceptibility of individuals to a disease;

ii. The course or natural history of a disease;

iii. The response of a patient with a disease to a medical intervention, such as, for example, a drug, a biologic substance, physical energy such as radiation therapy, or a specific dietary regimen. The ability to predict either beneficial or detrimental responses is medically useful.

Thus variation in any of these three parameters may constitute the basis for initiating a pharmacogenetic study directed to the identification of the genetic sources of interpatient variation. The effect of a DNA sequence variance or variances on disease susceptibility or natural history (i and ii, above) are of particular interest as the variances can be used to define patient subsets which behave differently in response to medical interventions such as those described in (iii).

In other words, a variance can be useful for customizing medical therapy at least for either of two reasons. First, the variance may be associated with a specific disease subset that behaves differently with respect to one or more therapeutic interventions (i and ii above); second, the variance may affect response to a specific therapeutic intervention (iii above). Consider for exemplary purposes pharmacological therapeutic interventions. In the first case, there may be no effect of a particular gene sequence variance on the observable pharmacological action of a drug, yet the disease subsets defined by the variance or variances differ in their response to the drug because, for example, the drug acts on a pathway that is more relevant to disease pathophysiology in one variance-defined patient subset thanin another variance-defined patient subset. The second type of useful gene sequence variance affects the pharmacological action of a drug or other treatment. Effects on pharmacological responses fall generally into two categories; pharmacokinetic and pharmacodynamic effects. These effects have been defined as follows in Goodman and Gilman's Phamacologic Basis of Therapeutics (ninth edition, McGraw Hill, New York, 1986): "Pharmacokinetics" deals with the absorption, distribution, biotransformations and excretion of drugs. The study of the biochemical and physiological effects of drugs and their mechanisms of action is termed "pharmacodynamics."

Useful gene sequence variances for this invention can be described as variances which partition patients into two or more groups that respond differently to a therapy, regardless of the reason for the difference, and regardless of whether the reason for the difference is known.

B. Identification of Specific Genes and Correlation of Variances in Those Genes with Response to Treatment of Diseases or Conditions

It is useful to identify particular genes which do or are likely to mediate the efficacy or safety of a treatment method for a disease or condition, particularly in view of the large number of genes which have been identified and which continue to be identified in humans. As is further discussed in section C below, this correlation can proceed by different paths. One exemplary method utilizes prior information on the pharmacology or pharmacokinetics or pharmacodynamics of a treatment method, e.g., the action of a drug, which indicates that a particular gene is, or is likely to be, involved in the action of the treatment method, and further suggests that variances in the gene may contribute to variable response to the treatment method.

Alternatively, if such information is not known, variances in a gene can be correlated empirically with treatment response. In this method, variances in a gene which exist in a population can be identified. The presence of the different variances or haplotypes in individuals of a study group, which is preferably representative of a population or populations, is determined. This variance information is then correlated with treatment response of the various individuals as an indication that genetic variability in the gene is at least partially responsible for differential treatment response. Statistical measures known to those skilled in the art are preferably used to measure the fraction of interpatient variation attributable to any one variance.

Useful methods for identifying genes relevant to the physiologic action of a drug or other treatment are known to those skilled in the art, and include large scale analysis of gene expression in cells treated with the drug compared to control cells, or large scale analysis of the protein expression pattern in treated vs. untreated cells, or the use of techniques for identification of interacting proteins or ligand-protein interactions.

C. Development of a Diagnostic Test to Determine Variance Status

In accordance with the description in the Summary above, the present invention generally concerns the identification of variances in genes which are indicative of the effectiveness of a treatment in a patient. The identification of specific variances, in effect, can be used as a diagnostic or prognostic test. Correlation of treatment efficacy and/or toxicity with particular genes and gene families or pathways is provided in Stanton et al., U.S. Provisional Application 60/093,484, filed July 20, 1998, entitled GENE SEQUENCE VARIANCES WITH UTILITY IN DETERMINING THE TREATMENT OF DISEASE (concerns the safety and efficacy of compounds active on folate or pyrimidine metabolism or action).

Genes identified in the examples below and the attached Tables and Figures can be used in the present invention.

Methods for diagnostic tests are well known in the art. Generally in this invention, the diagnostic test involves determining whether an individual has a variance or variant form of a gene that is involved in the disease or condition or the action of the drug or other treatment or effects of such treatment. Such a variance or variant form of the gene is preferably one of several different variances or forms of the gene that have been identified within the population and are known to be present at a certain frequency. In an exemplary method, the diagnostic test involves performed by amplifying a segment of DNA or RNA (generally after converting the RNA to cDNA) spanning one or more variances in the gene sequence. Preferably, the amplified segment is <500 bases in length, in an alternative embodiment the amplified segment is <100 bases in length, most preferably <45 bases in length. In many cases, the diagnostic test is performed by amplifying a segment of DNA or RNA (cDNA) spanning a variance, or even spanning more than one variance in the gene sequence and preferably maintaining the phase of the variances on each allele. The term "phase" means the association of variances on a single copy of the gene, such as the copy transmitted from the mother (maternal copy or maternal allele) or the father (paternal copy or paternal allele). It is apparent that such diagnostic tests are performed after initial identification of variances within the gene.

Diagnostic genetic tests useful for practicing this invention belong to two types: genotyping tests and haplotyping tests. A genotyping test simply provides the status of a variance or variances in a subject or patient. For example suppose nucleotide 150 of hypothetical gene X on an autosomal chromosome is an adenine (A) or a guanine (G) base. The possible genotypes in any individual are AA, AG or GG at nucleotide 150 of gene X.

In a haplotyping test there is at least one additional variance in gene X, say at nucleotide 810, which varies in the population as cytosine (C) or thymine (T). Thus a particular copy of gene X may have any of the following combinations of nucleotides at positions 150 and 810: 150A-810C, 150A-810T, 150G-810C or 150G-810T. Each of the four possibilities is a unique haplotype. If the two nucleotides interact in either RNA or protein, then knowing the haplotype can be important. The point of a haplotyping test is to determine the haplotypes present in a DNA or cDNA sample (e.g. from a patient). In the example provided there are only four possible haplotypes, but, depending on the number of variances in the gene and their distribution in human populations there may be three, four, five, six or more haplotypes at a given gene. The most useful haplotypes for this invention are those which occur commonly in the population being treated for a disease or condition. Preferably such haplotypes occur in at least 5% of the population, more preferably in at least 10%, still more preferably in at least 20% of the population and most preferably in at least

30% or more of the population. Conversely, when the goal of a pharmacogenetic program is to identify a relatively rare population that has an adverse reaction to a treatment, the most useful haplotypes may be rare haplotypes, which may occur in less than 5%, less than 2%, or even in less than 1% of the population. One skilled in the art will recognize that the frequency of the adverse reaction will provide a useful guide to the likely frequency of salient causative haplotypes.

Based on the identification of variances or variant forms of a gene, a diagnostic test utilizing methods known in the art can be used to determine whether a particular form of the gene, containing specific variances or haplotypes, or combinations of variances and haplotypes, is present in at least one copy, one copy, or more than one copy in an individual. Such tests are commonly performed using DNA or RNA collected from blood, cells, tissue scrapings or other cellular materials, and can be performed by a variety of methods including, but not limited to, hybridization with allele-specific probes, enzymatic mutation detection, chemical cleavage of mismatches, mass spectrometry or DNA sequencing, including minisequencing. Methods for haplotyping are provided in this application. In particular embodiments, hybridization with allele specific probes can be conducted in two formats: (1) allele specific oligonucleotides bound to a solid phase (glass, silicon, nylon membranes) and the labelled sample in solution, as in many DNA chip applications, or (2) bound sample (often cloned DNA or PCR amplified DNA) and labelled oligonucleotides in solution (either allele specific or short so as to allow sequencing by hybridization). The application of such diagnostic tests is possible after identification of variances that occur in the population. Diagnostic tests may involve a panel of variances from one or more genes, often on a solid support, which enables the simultaneous determination of more than one variance in one or more genes.

D. Use of Variance Status to Determine Treatment

The present disclosure describes exemplary gene sequence variances in genes identified in a gene table herein (e.g., Tables 2, 6, and 8), and variant forms of these gene that may be determined using diagnostic tests. As indicated in the Summary, such a variance-based diagnostic test can be used to determine whether or not to administer a specific drug or other treatment to a patient for treatment of a disease or condition. Preferably such diagnostic tests are incorporated in texts such as Clinical Diagnosis and Management by Laboratory Methods (19th Ed) by John B. Henry (Editor) W B Saunders Company, 1996; Clinical Laboratory Medicine : Clinical Application of Laboratory Data, (6th edition) by R. Ravel, Mosby-Year Book, 1995, or medical textbooks including, without limitation, textbooks of medicine, laboratory medicine, therapeutics, pharmacy, pharmacology, nutrition, allopathic, homeopathic, and osteopathic medicine; most preferably such a diagnostic test is specified by regulatory authorities, e.g., by the U.S. *Food and Drug*

Administration, and is incorporated in the label or insert as well as the Physicians Desk Reference.

In such cases, the procedure for using the drug is restricted or limited on the basis of a diagnostic test for determining the presence of a variance or variant form of a gene. The procedure may include the route of administration of the drug, the dosage form, dosage, schedule of administration or use with other drugs; any or all of these may require selecting or determination consistent with the results of the diagnostic test or a plurality of such tests. Preferably the use of such diagnostic tests to determine the procedure for administration of a drug is incorporated in a text such as those listed above, or medical textbooks, for example, textbooks of medicine, laboratory medicine, therapeutics, pharmacy, pharmacology, nutrition, allopathic, homeopathic, and osteopathic medicine. As previously stated, preferably such a diagnostic test or tests are required by regulatory authorities and are incorporated in the label or insert as well as the Physicians Desk Reference.

Variances and variant forms of genes useful in conjunction with treatment methods may be associated with the origin or the pathogenesis of a disease or condition. In many useful cases, the variant form of the gene is associated with a specific characteristic of the disease or condition that is the target of a treatment, most preferably response to specific drugs or other treatments. Examples of diseases or conditions ameliorable by the methods of this invention are identified in the Examples and tables below; in general treatment of disease with current methods, particularly drug treatment, always involves some unknown element (involving efficacy or toxicity or both) that can be reduced by appropriate diagnostic methods.

Alternatively, the gene is involved in drug action, and the variant forms of the gene are associated with variability in the action of the drug. For example, in some cases, one variant form of the gene is associated with the action of the drug such that the drug will be effective in an individual who inherits one or two copies of that form of the gene. Alternatively, a variant form of the gene is associated with the action of the drug such that the drug will be toxic or otherwise contra-indicated in an individual who inherits one or two copies of that form of the gene.

In accord with this invention, diagnostic tests for variances and variant forms of genes as described above can be used in clinical trials to demonstrate the safety and efficacy of a drug in a specific population. As a result, in the case of drugs which show variability in patient response correlated with the presence or absence of a variance or variances, it is preferable that such drug is approved for sale or use by regulatory agencies with the recommendation or requirement that a diagnostic test be performed for a specific variance or variant form of a gene which identifies specific populations in which the drug will be safe and/or effective. For example, the drug may be approved for sale or use by regulatory

agencies with the specification that a diagnostic test be performed for a specific variance or variant form of a gene which identifies specific populations in which the drug will be toxic. Thus, approved use of the drug, or the procedure for use of the drug, can be limited by a diagnostic test for such variances or variant forms of a gene; or such a diagnostic test may be considered good medical practice, but not absolutely required for use of the drug.

As indicated, diagnostic tests for variances as described in this invention may be used in clinical trials to establish the safety and efficacy of a drug. Methods for such clinical trials are described below and/or are known in the art and are described in standard textbooks. For example, diagnostic tests for a specific variance or variant form of a gene may be incorporated in the clinical trial protocol as inclusion or exclusion criteria for enrollment in the trial, to allocate certain patients to treatment or control groups within the clinical trial or to assign patients to different treatment cohorts. Alternatively, diagnostic tests for specific variances may be performed on all patients within a clinical trial, and statistical analysis performed comparing and contrasting the efficacy or safety of a drug between individuals with different variances or variant forms of the gene or genes. Preferred embodiments involving clinical trials include the genetic stratification strategies, phases, statistical analyses, sizes, and other parameters as described herein.

Similarly, diagnostic tests for variances can be performed on groups of patients known to have efficacious responses to the drug to identify differences in the frequency of variances between responders and non-responders. Likewise, in other cases, diagnostic tests for variance are performed on groups of patients known to have toxic responses to the drug to identify differences in the frequency of the variance between those having adverse events and those not having adverse events. Such outlier analyses may be particularly useful if a limited number of patient samples are available for analysis. It is apparent that such clinical trials can be or are performed after identifying specific variances or variant forms of the gene in the population.

The identification and confirmation of genetic variances is described in certain patents and patent applications. The description therein is useful in the identification of variances in the present invention. For example, a strategy for the development of anticancer agents having a high therapeutic index is described in Housman, International Application PCT/US/94 08473 and Housman, INHIBITORS OF ALTERNATIVE ALLELES OF GENES ENCODING PROTEINS VITAL FOR CELL VIABILITY OR CELL GROWTH AS A BASIS FOR CANCER THERAPEUTIC AGENTS, U.S. Patent 5,702,890, issued December 30, 1997, which are hereby incorporated by reference in their entireties. Also, a number of gene targets and associated variances are identified in Housman et al., U.S. Patent Application 09/045,053, entitled TARGET ALLELES FOR ALLELE-SPECIFIC DRUGS, filed March 19, 1998, which is hereby incorporated by

reference in its entirety, including drawings.

The described approach and techniques are applicable to a variety of other diseases, conditions, and/or treatments and to genes associated with the etiology and pathogenesis of such other diseases and conditions and the efficacy and safety of such other treatments.

Useful variances for this invention can be described generally as variances which partition patients into two or more groups that respond differently to a therapy (a therapeutic intervention), regardless of the reason for the difference, and regardless of whether the reason for the difference is known.

II. From Variance List to Clinical Trial: Identifying Genes and Gene Variances that Account for Variable Responses to Treatment

There are a variety of useful methods for identifying a subset of genes from a large set that should be prioritized for further investigation with respect to their influence on inter-individual variation in disease predisposition or response to a particular drug. These methods include for example, (1) searching the relevant literature to identify genes relevant to a disease or the action of a drug; (2) screening the genes identified in step 1 for variances. A large set of exemplary variances are provided in Tables 3, 4, 10, and 11; (3) using computational tools to predict the functional effects of variances in specific genes; (4) using *in vitro* or *in vivo* experiments to identify genes which may participate in the response to a drug or treatment, and to determine the variances which affect gene, RNA or protein function, and may therefore be important genetic variables affecting disease manifestations or drug response; and (5) retrospective or prospective clinical trials. Each of these methods is considered below in some detail.

(1) To begin, one preferably identifies, for a given treatment, a set of candidate genes that are likely to affect disease phenotype or drug response. This can be accomplished most efficiently by first assembling the relevant medical, pharmacological and biological data from available sources (e.g., public databases and publications). One skilled in the art can review the literature (textbooks, monographs, journal articles) and online sources (databases) to identify genes most relevant to the action of a specific drug or other treatment, particularly with respect to its utility for treating a specific disease, as this beneficially allows the set of genes to be analyzed ultimately in clinical trials to be reduced from an initial large set. Specific strategies for conducting such searches are described below. In some instances the literature may provide adequate information to select genes to be studied in a clinical trial, but in other cases additional experimental investigations of the sort described below will be preferable to maximize the likelihood that the salient genes and variances are moved forward into clinical studies.

Experimental data are also useful in establishing a list of candidate genes, as described below.

(2) Having assembled a list of candidate genes generally the second step is to screen for variances in each candidate gene. Experimental and computational methods for variance detection are described in this invention, and a tables of exemplary variances is provided (e.g., Table 3, 4, 10, and 11) as well as methods for identifying additional variances.

(3) Having identified variances in candidate genes the next step is to assess their likely contribution to clinical variation in patient response to therapy, preferably by using informatics-based approaches such as DNA and protein sequence analysis and protein modeling. The literature and informatics-based approaches provide the basis for prioritization of candidate genes, however it may in some cases be desirable to further narrow the list of candidate genes, or to measure experimentally the phenotype associated with specific variances or sets of variances (e.g. haplotypes).

(4) Thus, as a third step in candidate gene analysis, one skilled in the art may elect to perform *in vitro* or *in vivo* experiments to assess the functional importance of gene variances, using either biochemical or genetic tests. (Certain kinds of experiments – for example gene expression profiling and proteome analysis - may not only allow refinement of a candidate gene list but may also lead to identification of additional candidate genes.) Combination of two or all of the three above methods will provide sufficient information to narrow the set of candidate genes and variances to a number that can be studied in a clinical trial with adequate statistical power.

(5) The fourth step is to design retrospective or prospective human clinical trials to test whether the identified allelic variance, variances, or haplotypes or combination thereof influence the efficacy or toxicity profiles for a given drug or other therapeutic intervention. It should be recognized that this fourth step is the crucial step in producing the type of data that would justify introducing a diagnostic test for at least one variance into clinical use. Thus while each of the above four steps are useful in particular instances of the invention, this final step is indispensable. Further guidance and examples of how to perform these five steps is provided below.

1. Identification of Candidate Genes Relevant to the Action of a Drug

Practice of this invention will often begin with identification of a specific pharmaceutical product, for example a drug, that would benefit from improved efficacy or reduced toxicity or both, and the recognition that pharmacogenetic investigations as described herein provide a basis for achieving such improved characteristics. The question then becomes which of the genes and variances provided in this application, e.g., in Tables 3, 4, 10, and 11, would be most relevant to interpatient variation in response to the drug. As

discussed above, the set of relevant genes includes both genes involved in the disease process and genes involved in the interaction of the patient and the treatment – for example genes involved in pharmacokinetic and pharmacodynamic action of a drug. The biological and biomedical literature and online databases provide useful guidance in selecting such genes. Specific guidance in the use of these resources is provided below.

Review the literature and online sources

One way to find genes that affect response to a drug in a particular disease setting is to review the published literature and available online databases regarding the pathophysiology of the disease and the pharmacology of the drug. Literature or online sources can provide specific genes involved in the disease process or drug response, or describe biochemical pathways involving multiple genes, each of which may affect the disease process or drug response.

Alternatively, biochemical or pathological changes characteristic of the disease may be described; such information can be used by one skilled in the art to infer a set of genes that can account for the biochemical or pathologic changes. For example, to understand variation in response to a drug that modulates serotonin levels in a central nervous system (CNS) disorder associated with altered levels of serotonin one would preferably study, at a minimum, variances in genes responsible for serotonin biosynthesis, release from the cell, receptor binding, presynaptic reuptake, and degradation or metabolism. Genes responsible for each of these functions should be examined for variation that may account for interpatient differences in drug response or disease manifestations. As recognized by those skilled in the art, a comprehensive list of such genes can be obtained from textbooks, monographs and the literature.

There are several types of scientific information, described in some detail below, that are valuable for identifying a set of candidate genes to be investigated with respect to a specific disease and therapeutic intervention. First there is the medical literature, which provides basic information on disease pathophysiology and therapeutic interventions. A subset of this literature is devoted to specific description of pathologic conditions. Second there is the pharmacology literature, which will provide additional information on the mechanism of action of a drug (pharmacodynamics) as well as its principal routes of metabolic transformation (pharmacokinetics) and the responsible proteins. Third there is the biomedical literature (principally genetics, physiology, biochemistry and molecular biology), which provides more detailed information on metabolic pathways, protein structure and function and gene structure. Fourth, there are a variety of online databases that provide additional information on metabolic pathways, gene families, protein function and other subjects relevant to selecting a set of genes that are likely to affect the response to a

treatment.

Medical Literature

A good starting place for information on molecular pathophysiology of a specific disease is a general medical textbook such as Harrison's Principles of Internal Medicine, 14th edition, (2 Vol Set) by A.S. Fauci, E. Braunwald, K.J. Isselbacher, et al. (editors), McGraw Hill, 1997, or Cecil Textbook of Medicine (20th Ed) by R. L. Cecil, F. Plum and J. C. Bennett (Editors) W B Saunders Co., 1996. For pediatric diseases texts such as Nelson Textbook of Pediatrics (15th edition) by R.E. Behrman, R.M. Kliegman, A.M. Arvin and W.E. Nelson (Editors), W B Saunders Co., 1995 or Oski's Principles and Practice of Pediatrics (3rd Edition) by J.A. Mamillan & F.A. Oski Lippincott-Raven, 1999 are useful introductions. For obstetrical and gynecological disorders texts such as Williams Obstetrics (20th Ed) by F.G. Cunningham, N.F. Gant, P.C. McDonald et al. (Editors), Appleton & Lange, 1997 provide general information on disease pathophysiology. For psychiatric disorders texts such as the Comprehensive Textbook of Psychiatry, VI (2 Vols) by H.I. Kaplan and B.J. Sadock (Editors), Lippincott, Williams & Wilkins, 1995, or The American Psychiatric Press Textbook of Psychiatry (3rd edition) by R.E. Hales, S.C. Yudofsky and J.A. Talbott (Editors) Amer Psychiatric Press, 1999 provide an overview of disease nosology, pathophysiological mechanisms and treatment regimens.

In addition to these general texts, there are a variety of more specialized medical texts that provide greater detail about specific disorders which can be utilized in developing a list of candidate genes and variances relevant to interpatient variation in response to a treatment. For example, within the field of medicine there are standard textbooks for each of the subspecialties. Some specific examples include:

Heart Disease: A Textbook of Cardiovascular Medicine (2 Volume set) by E. Braunwald (Editor), W B Saunders Co., 1996.

Hurst's the Heart, Arteries and Veins (9th Ed) (2 Vol Set) by R.W. Alexander, R.C. Schlant, V. Fuster, W. Alexander and E.H. Sonnenblick (Editors) McGraw Hill, 1998.

Principles of Neurology (6th edition) by R.D. Adams, M. Victor (editors), and A.H. Ropper (Contributor), McGraw Hill, 1996.

Sleisenger & Fordtran's Gastrointestinal and Liver Disease: Pathophysiology, Diagnosis, Management (6th edition) by M. Feldman, B.F. Scharschmidt and M. Sleisenger (Editors), W B Saunders Co., 1997.

Textbook of Rheumatology (5th edition) by W.N. Kelley, S. Ruddy, E.D. Harris Jr. and C.B. Sledge (Editors) (2 volume set) W B Saunders Co., 1997.

Williams Textbook of Endocrinology (9th edition) by J.D. Wilson, D.W. Foster, H. M. Kronenberg and Larsen (Editors), W B Saunders Co., 1998.

Wintrobe's Clinical Hematology (10th Ed) by G.R. Lee, J. Foerster (Editor) and J. Lukens (Editors) (2 Volumes) Lippincott, Williams & Wilkins, 1998.

Cancer: Principles & Practice of Oncology (5th edition) by V.T. Devita, S.A. Rosenberg and S. Hellman (editors), Lippincott-Raven Publishers, 1997.

Principles of Pulmonary Medicine (3rd edition) by S.E. Weinberger & J Fletcher (Editors), W B Saunders Co., 1998.

Diagnosis and Management of Renal Disease and Hypertension (2nd edition) by A.K. Mandal & J.C. Jennette (Editors), Carolina Academic Press, 1994.Massry & Glassock's Textbook of Nephrology (3rd edition) by S.G. Massry & R.J. Glassock (editors) Williams & Wilkins, 1995.

The Management of Pain by J.J. Bonica, Lea and Febiger, 1992

Ophthalmology by M. Yanoff & J.S. Duker, Mosby Year Book, 1998

Clinical Ophthalmology: A Systemic Approach by J.J. Kanski, Butterworth-Heineman, 1994.Essential Otolaryngology by J.K. Lee Appleton and Lange 1998.

In addition to these subspecialty texts there are many textbooks and monographs that concern more restricted disease areas, or specific diseases. Such books provide more extensive coverage of pathophysiologic mechanisms and therapeutic options. The number of such books is too great to provide examples for all but a few diseases, however one skilled in the art will be able to readily identify relevant texts. One simple way to search for relevant titles is to use the search engine of an online bookseller such as http://www.amazon.com or http://www.barnesandnoble.com using the disease or drug (or the group of diseases or drugs to which they belong) as search terms. For example a search for asthma would turn up titles such as Asthma : Basic Mechanisms and Clinical Management (3rd edition) by P.J. Barnes, I.W. Rodger and N.C. Thomson (Editors), Academic Press, 1998 and Airways and Vascular Remodelling in Asthma and Cardiovascular Disease : Implications for Therapeutic Intervention : Based on the Scientific Program, by C. Page & J. Black (Editors), Academic Press, 1994.

Pathology Literature

In addition to medical texts there are texts that specifically address disease etiology and pathologic changes associated with disease. A good general pathology text is Robbins Pathologic Basis of Disease (6th edition) by R.S. Cotran, V. Kumar, T. Collins and S.L. Robbins, W B Saunders Co., 1998. Specialized pathology texts exist for each organ system and for specific diseases, similar to medical texts. These texts are useful sources of information for one skilled in the art for developing lists of genes that may account for some of the known pathologic changes in disease tissue. Exemplary texts are as follows:

Bone Marrow Pathology 2nd edition, by B.J. Bain, I. Lampert. & D. Clark, Blackwell Science, 1996

Atlas of Renal Pathology by F.G. Silva, W.B. Saunders, 1999.

Fundamentals of Toxicologic Pathology by W.M. Haschek and C.G. Rousseaux, Academic Press, 1997.

Gastrointestinal Pathology by P. Chandrasoma, Appleton and Lange, 1998.

Ophthalmic Pathology with Clinical Correlations by J. Sassani, Lippincott-Raven, 1997.

Pathology of Bone and Joint Disorders by F. McCarthy, F.J. Frassica and A. Ross, W. B. Saunders, 1998.

Pulmonary Pathology by M.A. Grippi, Lippicott-Raven, 1995.

Neuropathology by D. Ellison, L. Chimelli, B. Harding, S. Love& J. Lowe, Mosby Year Book, 1997.

Greenfield's Neuropatholgy 6th edition by J.G. Greenfield, P.L. Lantos & D.I. Graham, Edward Arnold, 1997.

Pharmacology, Pharmacogenetics and Pharmacy Literature

There are also both general and specialized texts and monographs on pharmacology that provide data on pharmacokinetics and pharmacodynamics of drugs. The discussion of pharmacodynamics (mechanism of action of the drug)in such texts is often supported by a review of the biochemical pathway or pathways that are affected by the drug. Also, proteins related to the target protein are often listed; it is important to account for variation in such proteins as the related proteins may be involved in drug pharmacology. For example, there are 14 known serotonin receptors. Various pharmacological serotonin agonists or antagonists have different affinities for these different receptors. Variation in a specific receptor may affect the pharmacology not only of drugs intentionally targeted to that receptor, but also drugs targeted to different receptors, that may have differential action on two allelic forms of the non-targeted receptor. Thus genes encoding proteins structurally related to the target protein are useful for screening for variance in the present invention. A good general pharmacology text is Goodman & Gilman's the Pharmacological Basis of Therapeutics (9th Ed) by J.G. Hardman, L.E. Limbird, P.B. Molinoff, R.W. Ruddon and A.G. Gilman (Editors) McGraw Hill, 1996. There are also texts that focus on the pharmacology of drugs for specific disease areas, or specific classes of drugs (e.g. natural products) or adverse drug interactions, among other subjects. Specific examples include:

The American Psychiatric Press Textbook of Psychopharmacology (2nd edition) by A.F. Schatzberg & C.B. Nemeroff (Editors), Amer Psychiatric Press, 1998. ISBN: 0880488174

Essential Psychopharmacology : Neuroscientific Basis and Practical Applications by

N. Muntner and S.M. Stahl, Cambridge Univ Press, 1996.

There are also texts on pharmacogenetics which are particularly useful for identifying genes which may contribute to variable pharmacokinetic response. In addition there are texts on some of the major xenobiotic metabolizing proteins, such as the cytochrome P450 genes.

Pharmacogenetics of Drug Metabolism (International Encyclopedia of Pharmacology and Therapeutics) by Werner Kalow (Editor) Pergamon Press, 1992.

Genetic Factors in Drug Therapy : Clinical and Molecular Pharmacogenetics by D.A Price Evans, Cambridge Univ Press, 1993.

Pharmacogenetics (Oxford Monographs on Medical Genetics, 32) by W.W. Weber, Oxford Univ Press, 1997.

Cytochrome P450 : Structure, Mechanism, and Biochemistry by P.R. Ortiz de Montellano (Editor), Plenum Publishing Corp, 1995.

Appleton & Lange's Review of Pharmacy, 6th edition, (Appleton & Lange's Review Series) by G.D. Hall & B.S. Reiss, Appleton & Lange, 1997.

Genetics, Biochemistry and Molecular Biology Literature

In addition to the medical, pathology, and pharmacology texts listed above there are several information sources that one skilled in the art will turn to for information on the genetic, physiologic, biochemical, and molecular biological aspects of the disease, disorder or condition or the effect of the therapeutic intervention on specific physiologic processes. The biomedical literature may include information on nonhuman organisms that is relevant to understanding the likely disease or pharmacological pathways in man.

Genetic texts may provide insight into the likely effect of an allelic variance, variances, or haplotypes on individual responses to a therapeutic intervention, particularly if there are genetic variances known to effect drug response. Example 1 describes variances in the dihydropyrimidine dehydrogenase (DPD) gene locus and their effects on fluoropyrimidine catabolism. DPD is an example of a gene that, in rare mutant forms, is associated with severe fluoropyrimidine poisoning. It is reasonable to expect that more common alleles may exist at the DPD locus and may affect fluoropyrimidine metabolism, thus accounting for interpatient variation. Thus the genetics of a rare allele or alleles may provide a basis for examining the effects of commonly occuring alleles on moderate phenotypes. The genetics of rare DPD deficiency is well described in medical genetics textbooks listed below, for example see Scriver et al (full citation below).

Also provided below are illustrative texts which will aid in the identification of a pathway or pathways, and a gene or genes that may be relevant to interindividual variation in response to a therapy. Textbooks of biochemistry, genetics and physiology are often useful

sources for such pathway information. In order to ascertain the appropriate methods to analyze the effects of an alleleic variance, variances, or haplotypes in vitro, one skilled in the art will review existing information on molecular biology, cell biology, genetics, biochemistry; and physiology. Such texts are useful sources for general and specific information on the genetic and biochemical processes involved in disease and in drug action, as well as experimental procedures that may be useful in performing in vitro research on an allelic variance, variances, or haplotye.

Texts on gene structure and function and RNA biochemistry will be useful in evaluating the consequences of variances that do not change the coding sequence. Such variances may alter the interaction of RNA with proteins or other regulatory molecules affecting RNA processing, polyadenylation, and export.

Molecular and Cellular Biology

Molecular Cell Biology by H. Lodish, D. Baltimore, A. Berk, L. Zipurksy & J. Darnell, W H Freeman & Co., 1995.

"Essentials of Molecular Biology", D. Freifelder and MalacinskiJones and Bartlett, 1993.

"Genes and Genomes: A Changing Perspective", M. Singer and P. Berg, 1991. University Science Books

"Gene Structure and Expression", J.D. Hawkins, 1996. Cambridge University Press

Molecular Biology of the Cell, 2nd edition, B. Alberts et alGarland Publishing, 1994.,

Molecular Genetics

The Metabolic and Molecular Bases of Inherited Disease by C. R. Scriver, A.L. Beaudet, W.S. Sly (Editors), 7th edition, McGraw Hill, 1995

"Genetics and Molecular Biology", R. Schleif, 1994. 2nd edition, Johns Hopkins University Press

"Genetics", P.J. Russell, 1996. 4th edition, Harper Collins

"An Introduction to Genetic Analysis", Griffiths et al.1993. 5th edition, W.H. Freeman and Company

"Understanding Genetics: A molecular approach", Rothwell, 1993. Wiley-Liss

General Biochemistry

"Biochemistry", L. Stryer, 1995. W.H. Freeman and Company

"Biochemistry", D. Voet and J.G. Voet, 1995. John Wiley and Sons

"Principles of Biochemistry", A.L. Lehninger, D.L. Nelson, and M.M. Cox, 1993. Worth Publishers

"Biochemistry", G. Zubay, 1998. Wm. C. Brown Communications

"Biochemistry", C.K. Mathews and K.E. van Holde, 1990. Benjamin/Cummings

Transcription

"Eukaryotic Transcriptiuon Factors", D.S. Latchman, 1995. Academic Press

"Eukaryotic Gene Transcription", S. Goodbourn (ed.), 1996. Oxford University Press.

"Transcription Factors and DNA Replication", D.S. Pederson and N.H. Heintz, 1994. CRC Press/R.G. Landes Company

"Transcriptional Regulation", S.L. McKnight and K. Yamamoto (eds.), 1992. 2 volumes, Cold Spring Harbor Laboratory Press

RNA

"Control of Messenger RNA Stability", J. Belasco and G. Brawerman (eds.), 1993. Academic Press

"RNA-Protein Interactions", Nagai and Mattaj (eds.), 1994. Oxford University Press

"mRNA Metabolism and Post-transcriptional Gene Regulation", Harford and Morris (eds.), 1997. Wiley-Liss

Translation

"Translational Control", J.W.B. Hershey, M.B. Mathews, and N. Sonenberg (eds.), 1995. Cold Spring Harbor Laboratory Press

General Physiology

"Textbook of Medical Physiology" 9th Edtion by A.C. Guyton and J.E. Hall W.B. Saunders, 1997

"Review of Medical Physiology", 18th Edition by W.F. Ganong, Appleton and Lange, 1997

Online Databases

Those skilled in the art are familiar with how to search the literature, such as, e.g., libraries, online pubmed, abstract listings, and online mutation databases. One particularly useful resource is maintained at the web site of the National Center for Biotechnology Information (ncbi): http://www.ncbi.nlm.nih.gov/. From the ncbi site one can access Online Mendelian Inheritance in Man (OMIM). OMIM can be found at: http://www3.ncbi.nlm.nih.gov/Omim/searchomim.html. OMIM is a medically oriented database of genetic information with entries for thousands of genes. The OMIM record number is provided for many of the genes in Tables 10 and 11 (see column 3), and constitutes an excellent entry point for identification of references that point to the broader literature. Another useful site at NCBI is the Entrez browser, located at

http://www3.ncbi.nlm.nih.gov/Entrez/. One can search genomes, polynucleotides, proteins, 3D structures, taxonomy or the biomedical literature (PubMed) via the Entrez site. More generally links to a number of useful sites with biomedical or genetic data are maintained at sites such as Med Web at the Emory University Health Sciences Center Library: http://WWW.MedWeb.Emory.Edu/MedWeb/; Riken, a Japanese web site at: http://www.rtc.riken.go.jp/othersite.html with links to DNA sequence, structural, molecular biology, bioinformatics, and other databases; at the Oak Ridge National Laboratory web site: http://www.ornl.gov/hgmis/links.html; or at the Yahoo website of Diseases and Conditions: http://dir.yahoo.com/health/diseases_and_conditions/index.html. Each of the indicated web sites has additional useful links to other sites.

Another type of database with utility in selecting the genes on a biochemical pathway that may affect the response to a drug are databases that provide information on biochemical pathways. Examples of such databases include the Kyoto Encyclopedia of Genes and Genomes (KEGG), which can be found at: http://www.genome.ad.jp/kegg/kegg.html. This site has pictures of many biochemical pathways, as well as links to other metabolic databases such as the well known Boehringer Mannheim biochemical pathways charts: http://www.expasy.ch/cgi-bin/search-biochem-index. The metabolic charts at the latter site are comprehensive, and excellent starting points for working out the salient enzymes on any given pathway.

Each of the web sites mentioned above has links to other useful web sites, which in turn can lead to additional sites with useful information.

Research Libraries

Those skilled in the art will often require information found only at large libraries. The National Library of Medicine (http://www.nlm.nih.gov/) is the largest medical library in the world and its catalogs can be searched online. Other libraries, such as university or medical school libraries are also useful to conduct searches. Biomedical books such as those referred to above can often be obtained from online bookstores as described above.

Biomedical Literature

To obtain up to date information on drugs and their mechanism of action and biotransformation; disease pathophysiology; biochemical pathways relevant to drug action and disease pathophysiology; and genes that encode proteins relevant to drug action and disease one skilled in the art will consult the biomedical literature . A widely used, publically accessible web site for searching published journal articles is PubMed (http://www.ncbi.nlm.nih.gov/PubMed/). At this site, one can search for the most recent articles (within the last 1-2 months) or for specific details on methods that are less recent

(back to 1966). Many Journals also have their own sites on the world wide web and can be searched online. For example see the IDEAL web site at: http://www.apnet.com/www/ap/aboutid.html. This site is an online library, featuring full text journals from Academic Press and selected journals from W.B. Saunders and Churchill Livingstone. The site provides access (for a fee) to nearly 2000 scientific, technical, and medical journals.

Experimental methods for identification of genes involved in the action of a drug

There are a number of experimental methods for identifying genes and gene products that mediate or modulate the effects of a drug or other treatment. They encompass analyses of RNA and protein expression as well as methods for detecting protein – protein interactions and protein – ligand interactions. Two preferred experimental methods for identification of genes that may be involved in the action of a drug are (1) methods for measuring the expression levels of many mRNA transcripts in cells or organisms treated with the drug (2) methods for measuring the expression levels of many proteins in cells or organisms treated with the drug.

RNA transcripts or proteins that are substantially increased or decreased in drug treated cells or tissues relative to control cells or tissues are candidates for mediating the action of the drug. Other useful experimental methods include protein interaction methods such as the yeast two hybrid system and variants thereof which facilitate the detection of protein – protein interactions.

The pool of RNAs expressed in a cell is sometimes referred to as the transcriptome. Methods for measuring the transcriptome, or some part of it, are known in the art. A recent collection of articles summarizing some current methods appeared as a supplement to the journal *Nature Genetics*. (The Chipping Forecast. Nature Genetics supplement, volume 21, January 1999.) Experiments have been described in model systems that demonstrate the utility of measuring changes in the transcriptome before before and after changing the growth conditions of cells, for example by changing the nutritional status. The changes in gene expression help reveal the network of genes that mediate physiological responses to the altered growth condition. Similarly, the addition of a drug to the cellular or in vivo environment, followed by monitoring the changes in gene expression can aid in identification of pharmacological gene networks.

The pool of proteins expressed in a cell is sometimes referred to as the proteome. Studies of the proteome may include not only protein abundance but also protein subcellular localization and protein-protein interaction. Methods for measuring the proteome, or some part of it, are known in the art. One widely used method is to extract total cellular protein and separate it in two dimensions, for example first by size and then by isoelectric point.

The resulting protein spots can be stained and quantitated, and individual spots can be excised and analyzed by mass spectrometry to provide definitive identification. The results can be compared from two or more cell lines or tissues, at least one of which has been treated with a drug. The differential up or down modulation of specific proteins in response to drug treatment may indicate their role in mediating the pharmacologic actions of the drug. Another way to identify the network of proteins that mediate the actions of a drug is to exploit methods for identifying interacting proteins. By starting with a protein known to be involved in the action of a drug – for example the drug target – one can use systems such as the yeast two hybrid system and variants thereof (known to those skilled in the art) to identify additional proteins in the network of proteins that mediate drug action. The genes encoding such proteins would be useful for screening for DNA sequence variances, which in turn may be useful for analysis of interpatient variation in response to treatments. For example, the protein 5-lipoxygenase (5LO) s an enzyme which is a the beginning of the leukotriene biosynthetic pathway and is a target for anti-inflammatory drugs used to treat asthma and other diseases. In order to detect proteins that interact with 5-lipoxygenase the two-hybrid system was recently used to isolate three different proteins, none previously known to interact with 5LO. (Provost et al., Interaction of 5-lipoxygenase with cellular proteins. Proc. Natl. Acad. Sci. U.S.A. 96: 1881-1885, 1999.) A recent collection of articles summarizing some current methods in proteomics appeared in the August 1998 issue of the journal Electrophoresis (volume 19, number 11). Other useful articles include: Blackstock WP, et al. Proteomics: quantitative and physical mapping of cellular proteins. Trends Biotechnol. 17 (3): p. 121-7, 1999, and Patton W.F., Proteome analysis II. Protein subcellular redistribution: linking physiology to genomics via the proteome and separation technologies involved. *J. Chromatogr. B. Biomed. Sci. App.*. 722(1-2):203-23. 1999.

Since many of these methods can also be used to assess whether specific polymorphisms are likely to have biological effects, they should also be considered as relevant in section 3, below, concerning methods for assessing the likely contribution of variances in candidate genes to clinical variation in patient responses to therapy.

2. Screen for Variances in Genes that may be Related to Therapeutic Response

Having identified a set of genes that may affect response to a drug the next step is to screen the genes for variances that may account for interindividual variation in response to the drug. There are a variety of levels at which a gene can be screened for variances, and a variety of methods for variance screening. The two main levels of variance screening are genomic DNA screening and cDNA screening. Genomic variance detection may include screening the entire genomic segment spanning the gene from the transcription start site to the polyadenylation site. Alternatively genomic variance detection may (for intron containing genes) include the exons and some region around them containing the splicing

signals, for example, but not all of the intronic sequences. In addition to screening introns and exons for variances it is generally desirable to screen regulatory DNA sequences for variances. Promoter, enhancer, silencer and other regulatory elements have been described in human genes. The promoter is generally proximal to the transcription start site, although there may be several promoters and several transcription start sites. Enhancer, silencer and other regulatory elements may be intragenic or may lie outside the introns and exons, possibly at a considerable distance, such as 100 kb away. Variances in such sequences may affect basal gene expression or regulation of gene expression. In either case such variation may affect the response of an individual patient to a therapeutic intervention, for example a drug, as described in the examples. Thus in practicing the present invention it is useful to screen regulatory sequences as well as transcribed sequences, in order to identify variances that may affect gene transcription. Frequently information on the genomic sequence of a gene can be found in the sources above, particularly by searching GenBank or Medline (PubMed). The name of the gene can be entered at a site such as Entrez: http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html. Using the genomic sequence and information from the biomedical literature one skilled in the art can perform a variance detection procedure such as those described in examples 14, 15 and 16.

Variance detection is often first performed on the cDNA of a gene for several reasons. First, available data on functional sequence variances suggests that variances in the transcribed portion of a gene are most likely to have functional consequences as they can affect the interaction of the transcript with a wide variety of cellular factors during the complex processes of transcription, processing and translation. Second, as a practical matter the cDNA sequence of a gene is often available before the genomic structure is known, although the reverse may be true in the future as the sequence of the human genome is determined. If the genomic structure is not known then only the cDNA seqence can be scanned for variances. Methods for preparing cDNA are described in Example 13. Methods for variance detection on cDNA are described below and in the examples.

Methods for variance screening have been described, including DNA sequencing. See for example: US5698400: Detection of mutation by resolvase cleavage; US5217863: Detection of mutations in nucleic acids; and US5750335: Screening for genetic variation, as well as the examples and references cited therein for examples of useful variance detection procedures. Detailed variance detection procedures are also described in examples 14, 15 and 16. One skilled in the art will recognize that depending on the specific aims of a variance detection project (number of genes being screened, number of individuals being screened, total length of DNA being screened) one of the above cited methods may be preferable to the others, or yet another procedure may be optimal. A preferred method of variance detection is chain terminating DNA sequencing using dye labeled primers, cycle

sequencing and software for assessing the quality of the DNA sequence as well as specialized software for calling heterozygotes. The use of such procedures has been described by Nickerson and colleagues. See for example: Rieder M.J., et al. Automating the identification of DNA variations using quality-based fluorescence re-sequencing: analysis of the human mitochondrial genome. *Nucleic Acids Res.* 26 (4):967-73, 1998, and: Nickerson D.A., et al. PolyPhred: automating the detection and genotyping of single nucleotide substitutions using fluorescence-based resequencing. *Nucleic Acids Res.* 25 (14):2745-51, 1997. Although the variances provided in tables 3, 4, 10, and 11 consist principally of cDNA variances, it is a part of this invention that detection of genomic variances is also a useful method for identification of variances that may account for interpatient variation in response to a therapy.

3. Assess the Likely Contribution of Variances in Candidate Genes to Clinical Variation in Patient Responses to Therapy

Once a set of genes likely to affect disease pathophysiology or drug action has been identified, and those genes have been screened for variances, said variances (e.g., provided in Tables 3, 4, 10, and 11) can be assessed for their contribution to variation in the pharmacological or toxicological phenotypes of interest. There are several methods which can be used in the present invention for assessing the medical and pharmaceutical implications of a DNA sequence variance. They range from computational methods to *in vitro* and/or *in vivo* experimental methods (discussed below), to prospective human clinical trials (see below), and also include a variety of other laboratory and clinical measures that can provide evidence of the medical consequences of a variance. In general, human clinical trials constitute the highest standard of proof that a variance or set of variances is useful for selecting a method of treatment, however, computational and *in vitro* data, or retrospective analysis of human clinical data may provide strong evidence that a particular variance will affect response to a given therapy. Moreover, at an early stage in the analysis when there are many possible hypotheses to explain interpatient variation in treatment response, the use of informatics-based approaches to evaluate the likely functional effects of specific variances is an efficient way to proceed.

Informatics-based approaches to the prediction of the likely functional effects of variances include DNA and protein sequence analysis (phylogenetic approaches and motif searching) and protein modeling (based on coordinates in the protein database, or pdb; see http://www.rcsb.org/pdb/). Such analyses can be performed quickly and inexpensively, and the results allow selection of certain genes for more extensive *in vitro* or *in vivo* studies (see below) or for more variance detection (see above) or both.

More specifically, the structure of many medically and pharmaceutically important proteins, or homologs of such proteins in other species, or examples of domains present in such proteins, is known. Further, there are increasingly powerful tools for modeling the structure of proteins with unsolved structure, particularly if there is a related (e.g., a homologous) protein with known structure. (For reviews see: Rost et al., Protein fold recognition by prediction-based threading, *J. Mol. Biol.* 270:471-480, 1997; Firestine et al., Threading your way to protein function, *Chem. Biol.* 3:779-783, 1996) There are also powerful methods for identifying conserved domains and vital amino acid residues of proteins of unknown structure by analysis of phylogenetic relationships. (Deleage et al., Protein structure prediction: Implications for the biologist, *Biochimie* 79:681-686, 1997; Taylor et al., Multiple protein structure alignment, *Protein Sci.* 3:1858-1870, 1994) These methods can permit the prediction of functionally important variances, either on the basis of structure or evolutionary conservation. For example, a crystal structure can reveal which amino acids comprise a small molecule binding site. The identification of a polymorphic amino acid variance in the topological neighborhood of such a site, and in particular, the demonstration that at least one variant form of the protein has a variant amino acid which impinges on the known small molecule binding pocket differently from another variant form, provides strong evidence that the variance affects the function of the protein. From this it follows that the interaction of the protein with a treatment method, such an administered drug, will also likely be altered. One skilled in the art will recognize that the application of computational tools to the identification of functionally consequential variances involves applying the knowledge and tools of medicinal chemistry and physiology to the analysis.

Phylogenetic approaches to understanding sequence variation are also useful. Thus if a sequence variance occurs at a nucleotide or encoded amino acid residue where there is usually little or no variation in homologs of the protein of interest from non-human species, particularly evolutionarily remote species, then the variance is more likely to affect function of the RNA or protein.

4. Perform *in vitro* or *in vivo* Experiments to Assess the Functional Importance of Gene Variances

The selection of an appropriate experimental program for testing the medical consequences of a variance may differ depending on the nature of the variance, the gene, and the disease. For example if there is already evidence that a protein is involved in the pharmacologic action of a drug, then the *in vitro* demonstration that an amino acid variance in the protein affects its biochemical activity is strong evidence that the variance will have an

effect on the pharmacology of the drug in patients, and therefore that patients with different variant forms of the gene may have different responses to the same dose of drug. If the variance is silent with respect to protein coding information, or if it lies in a noncoding portion of the gene (e.g., a promoter, an intron, or a 5'- or 3'-untranslated region) then the appropriate biochemical assay may be to assess mRNA abundance, half life, or translational efficiency. If, on the other hand, there is no substantial evidence that the protein encoded by a particular gene is relevant to drug pharmacology, then the appropriate test is a clinical study addressing the responses to therapy of two patient groups distinguished on the basis of one or more variances. This approach reflects the current reality that biologists do not sufficiently understand gene regulation and gene expression to consistently make accurate inferences about the consequences of DNA sequence variances.

Thus, if there is a reasonable hypothesis regarding the effect of a protein on the action of a drug, then the *in vitro* and *in vivo* approaches described below will usefully predict whether a given variance is therapeutically consequential. If, on the other hand, there is no evidence of such an effect, then the most appropriate test is the empirical clinical measure of efficacy (which requires no evidence or assumptions regarding the mechanism by which the variance may exert an effect on a therapy). Clinical studies may be performed either prospectively or retrospectively.

Experimental Methods: Genomic DNA Analysis

Variances in DNA may affect the basal transcription or regulated transcription of a gene locus. Such variances may be located in any part of the gene but are most likely to be located in the promoter region, the first intron, or in 5' or 3' flanking DNA, where enhancer or silencer elements may be located. Methods for analyzing transcription are well known to those skilled in the art and exemplary methods are described in some of the texts cited below. Transcriptional run off assay is one useful method. Detailed protocols for useful methods can be found in texts such as: Current Protocols in Molecular Biology edited by: F.M. Ausubel, R.Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, K. Struhl, John Wiley & Sons, Inc, 1999, or: Molecular Cloning: A Laboratory Manual by J. Sambrook, E.F. Fritsch and T Maniatis. 1989. 3 vols, 2nd edition, Cold Spring Harbor Laboratory Press

Experimental Methods: RNA Analysis

RNA variances may affect a wide range of processes including RNA splicing, polyadenylation, capping, export from the nucleus, interaction with translation intiation, elongation or termination factors, or the ribosome, or interaction with cellular factors including regulatory proteins, or factors that may affect mRNA half life. However, any effect of variances on RNA function should ultimately be measurable as an effect on RNA

levels – either basal levels or regulated levels or levels in some abnormal cell state. Therefore one preferred method for assessing the effect of RNA variances on RNA function is to measure the levels of RNA produced by different alleles in one or more conditions of cell or tissue growth. Said measuring can be done by conventional methods such as Northern blots or RNAase protection assays (kits available from Ambion, Inc.), or by methods such as the Taqman assay (developed by the Applied Biosystems Division of the Perkin Elmer Corporation), or by using arrays of oligonucleotides or arrays of cDNAs attached to solid surfaces. Systems for arraying cDNAs are available commercially from companies such as Nanogen and General Scanning. Complete systems for gene expression analysis are available from companies such as Molecular Dynamics. For recent reviews of the technology see the supplement to volume 21 of Nature Genetics entitled "The Chipping Forecast", especially articles beginning on pages 9, 15, 20 and 25.

Additional methods for analyzing the effect of variances on RNA include secondary structure probing, and direct measurement of half life or turnover. Secondary structure can be determined by techniques such as enzymatic probing (using enzymes such as T1, T2 and S1 nuclease), chemical probing or RNAase H probing using oligonucleotides. Some RNA structural assays can be performed *in vitro* or on cell extracts or on

Experimental Methods: Protein Analysis

There are a variety of experimental methods for investigating the effect of a variance on response of a patient to a treatment. The preferred method will depend on the availability of cells expressing a particular protein, and the feasibility of a cell-based assay vs. assays on cell extracts, on proteins produced in a foreign host, or on proteins prepared by *in vitro* translation.

For example, the methods and systems listed below can be utilized to demonstrate differential expression and/or activity, or in model system phenotype/genotype correlations.

For the determination of protein levels or protein activity one could utilize a variety of techniques. The in vitro protein activity can be determined by transcription or translation in bacteria, yeast, baculovirus, COS cells (transient), CHO, or study directly in human cells. Further, one could perform pulse chase for experiments for the determination of changes in protein stability (half life).

One skilled in the art could manipulate the cell assay to address grouping the cells by genotypes or phenotypes. For example, identification of cells with different genotypes (possibly including families) and phenotype may be performed using standardized laboratory molecular biological protocols. After identification and grouping, one skilled in the art could determine whether there exists a correlation between cellular genotype and cellular phenotype.

Advancing an experimental preclinical program may include testing these in vitro hypotheses in vivo, e.g. an animal model. For example, one skilled in the art would readily have the ability to create gene knockouts. In this case, an embryonic stem cell is genetically manipulated to be deficient in a given gene. More specifically, a DNA construct is created that will undergo homologous recombination when inserted into the said embryonic stem cell nucleus. After the recombination event has occurred, the targeted gene is effectively inactivated due to the insertion of sequence (usually a translation stop or a marker gene sequence). This can be accomplished in worms, drosophila, or mice. The species chosen will be conducive to attain maximal experimental results for the particular gene and the particular variance, variances, or haplotype. Once the knockout species is created the candidate therapeutic intervention can be administered to the animal and tested for effects on gene expression or effects of various gene deficiencies. In the case whereby the chosen cell is a lower eukaryote, e.g. yeast, genetic manipulation occurs via introduction of a DNA construct that will undergo homologous recombination to disrupt the endogenous gene or genes.

The methods described above are reviewed and compiled in the following list of texts.

General Molecular Biology Methods

"Molecular Biology: A project approach", S.J. Karcher, Fall 1995. Academic Press

"DNA Cloning: A Practical Approach", D.M. Glover and B.D. Hayes (eds). 1995. IRL/Oxford University Press. Vol. 1 - Core Techniques; Vol 2 - Expression Systems; Vol. 3 - Complex Genomes; Vol. 4 -Mammalian Systems.

"Short Protocols in Molecular Biology", Ausubel et al. October 1995. 3rd edition, John Wiley and Sons

Current Protocols in Molecular Biology Edited by: F.M. Ausubel, R.Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, K. Struhl, (Series Editior: V.B. Chanda), 1988

"Molecular Cloning: A laboratory manual", J. Sambrook, E.F. Fritsch. 1989. 3 vols, 2nd edition, Cold Spring Harbor Laboratory Press

Polymerase chain reaction (PCR)

"PCR Primer: A laboratory manual", C.W. Diffenbach and G.S. Dveksler (eds.), 1995. Cold Spring Harbor Laboratory Press

"The Polymerase Chain Reaction", K.B. Mullis et al. (eds.), 1994. Birkhauser

"PCR Strategies", M.A. Innis, D.H. Gelf, and J.J. Sninsky (eds.), 1995. Academic Press

General procedures for discipline specific studies

Current Protocols in Neuroscience Edited by: J. Crawley, C. Gerfen, R. McKay, M. Rogawski, D. Sibley, P. Skolnick, (Series Editor: G. Taylor), 1997

Current Protocols in Pharmacology Edited by: S. J. Enna / M. Williams, J.W. Ferkany, T. Kenakin, R.E. Porsolt, J.P. Sullivan, (Series Editor: G. Taylor),1998

Current Protocols in Protein Science Edited by: J.E. Coligan, B.M. Dunn, H.L. Ploegh, D.W. Speicher, P.T. Wingfield, (Series Editor: Virginia Benson Chanda), 1995

Current Protocols in Cell Biology Edited by: J.S. Bonifacino, M. Dasso, J. Lippincott-Schwartz, J.B. Harford, K.M. Yamada, (Series Editor: K. Morgan) 1999

Current Protocols in Cytometry Managing Editor: J.P. Robinson, Z. Darzynkiewicz (ed) / P. Dean (ed), A. Orfao (ed), P. Rabinovitch (ed), C. Stewart (ed), H. Tanke (ed), L. Wheeless (ed), (Series Editor: J. Paul Robinson), 1997

Current Protocols in Human Genetics Edited by: N.C. Dracopoli, J.L. Haines, B.R. Korf, D.T. Moir, C.C. Morton, C.E. Seidman, J.G. Seidman, D.R. Smith, (Series Editor: A. Boyle), 1994

Current Protocols in Immunology Edited by: J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W. Strober, (Series Editor: R. Coico), 1991

III. Clinical Trials

A clinical trial is the definitive test of the utility of a variance or variances for the selection of optimal therapy. Clinical trials require no knowledge of the biological function of the gene containing the variance or variances to be assessed, nor any knowledge of how the therapeutic intervention to be assessed works at a biochemical level; the question of the utility of a variance can be addressed at a purely phenomenological level. On the other hand, if there is information about either the biochemical basis of a therapeutic intervention or the biochemical effects of a variance, then a clinical trial can be designed to test a specific hypothesis.

Methods for performing clinical trials are well known in the art. (Guide to Clinical Trials by Bert Spilker, Raven Press, 1991; The Randomized Clinical Trial and Therapeutic Decisions by Niels Tygstrup (Editor), Marcel Dekker; Recent Advances in Clinical Trial Design and Analysis (Cancer Treatment and Research, Ctar 75) by Peter F. Thall (Editor) Kluwer Academic Pub, 1995. However, performing a clinical trial to test the genetic contribution to interpatient variation in drug response requires some additional design considerations, including defining what the genetic hypothesis is, how it is to be tested, how many patients will need to be enrolled to have adequate statistical power to measure an effect of a specified magnitude (power analysis), definition of primary and secondary

endpoints, and methods of statistical analysis, as well as other aspects. In the outline below some of the major types of genetic hypothesis testing, power analysis, statistical analysis, etc. are summarized. One skilled in the art will recognize that certain of the methods will be best suited to specific clinical situations, and that additional methods are known and can be used in particular instances.

A. *Performing a Clinical Trial*

As used herein, a "clinical trial" is the testing of a therapeutic intervention in a volunteer human population for the purpose of determining whether a therapeutic intervention is safe and/or efficacious in the human volunteer or patient population for a given disease, disorder, or condition. The analysis of safety and efficacy in genetically defined subgroups differing by at least one variance is of particular interest.

A "clinical study" is that part of a clinical trial that involves determination of the effect a candidate therapeutic intervention on human subjects. It includes clinical evaluations of physiologic responses including pharmacokinetic (absorption, distribution, bioavailability, and excretion) as well as pharmacodynamic (physiologic response and efficacy) parameters. A pharmacogenetic clinical study is a clinical study that involves testing of one or more specific hypotheses regarding the effect of a genetic variance or variances (or set of variances, i.e. haplotype or haplotypes) in enrolled subjects or patients on response to a therapeutic intervention. These hypotheses are articulated before the study in the form of primary or secondary endpoints. For example the endpoint may be that in a particular genetic subgroup the rate of objectively defined responses exceeds some predefined threshold.

For each clinical study to commence enrollment and proceed to treat subjects at a given institution, an application that describes in detail the scientific premise for the therapeutic intervention and the procedures involved in the study, including the endpoints and analytical methods to be used in evaluating the data must be reviewed and accepted by regulatory authorities at the level of the institution and the federal government (in the U.S.). In the U.S., there are two regulatory bodies that oversee conduct of clinical trials: an Institutional Review Board (IRB) and the United States Food and Drug Administration (US FDA). The European counterpart of the US FDA is the European Medicines Evaluation Agency (EMEA). Similar agencies exist in other countries.

An Institutional Review Board accepts and reviews applications for clinical trials that are to be conducted at the institution and are to include healthy volunteers or human subjects from a defined patient population that seeks medical, surgical, rehabilitative, or social services at that institution. The application includes document sections that provide the rationale for and describe the scope of the clinical study. For example, an application to

an IRB may include a clinical protocol, and informed consent forms.

It is also customary, but not required, to prepare an investigator's brochure which describes the scientific hypothesis for the proposed therapeutic intervention, the preclinical data, and the clinical protocol in concise language. The brochure is made available to any physician participating in the proposed or ongoing trial. The investigator's brochure for a pharmacogenetic clinical trial will include a full description of the genetic variance and/or variances believed or hypothesized to account for differential responses in the normal human subjects or patients, as well as a description of the genetic statistical analysis.

The supporting preclinical data is a report of all the *in vitro*, *in vivo* animal or previous human trial data that supports the safety and/or efficacy of a given therapeutic intervention. In a pharmacogenetic clinical trial the preclinical data may also include a description of the effect of a specific genetic variance or variances on biochemical or physiologic experimental variables, or on treatment outcomes, as determined by *in vitro* studies or by retrospective genetic analysis of clinical trial or other medical data (see below) used to first formulate or test a pharmacogenetic hypothesis.

The clinical protocol provides the relevant scientific and therapeutic introductory information, describes the inclusion and exclusion criteria for human subject enrollment, including genetic criteria if relevant, describes in detail the exact procedure or procedures for treatment using the candidate therapeutic intervention, describes laboratory analyses to be performed during the study period, and lastly describes the risks (both known and unknown) involving the use of the experimental candidate therapeutic intervention. In a clinical protocol for a pharmacogenetic clinical trial, the clinical protocol will further describe the gene or genes believed or hypothesized to affect differential patient responses and the variance or variances to be tested. Further, the clinical protocol for a pharmacogenetic clinical trial will include a description of the stratification of the treatment groups based on one or more gene sequence variances or combination of variances or haplotypes.

The informed consent document is a description of the therapeutic intervention and the clinical protocol in simple language (third grade level) for the patient to read, understand, and, if willing, agree to participate in the study by signing the document. In a pharmacogenetic clinical study the informed consent document will describe, in simple language, the use of a genetic test or a limited set of genetic tests to determine the subject or patients status at a particular gene variance or variances, and to further ascertain whether, in the study population, particular variances are associated with particular clinical or physiological responses.

The US FDA reviews proposed clinical trials through the process of an Investigational New Drug Application (IND). The IND is composed of the investigator's brochure, the supporting *in vitro* and *in vivo* animal or previous human data, the clinical

protocol, and the informed consent documents or forms. In each of the sections of the IND, a specific description of a single allelic variance or a number of variances to be tested in the clinical study will be included. For example, in the investigator's brochure a description of the gene or genes believed or hypothesized to account, at least in part, for differential responses will be included as well as a description of genetic variance or variances of a particular candidate gene or genes. Further, the preclinical data may include a description of *in vivo* or *in vitro* studies of the biochemical or physiologic effects of a variance or variances (e.g., haplotype) in a candidate gene or genes, as well as the predicted effects of the variance or variances on efficacy or toxicology of the candidate therapeutic intervention. Alternatively the results of retrospective genetic analysis of response data in patients treated with the candidate therapy may be the basis for formulating the genetic hypotheses to be tested in the prospective trial. For first in man clinical studies, the focus of this section will be safety. The US FDA reviews the application with a particular emphasis on the safety data and whether toxicological data is supportive and sufficient to justify proceeding to human testing.

The established phases of clinical development are Phase I, II, III, and IV. The fundamental objectives for each phase become increasingly complex as the stages of clinical development progress. In Phase I, safety in humans is the primary focus. In these studies, dose-ranging designs establish whether the candidate therapeutic intervention is safe in the suspected therapeutic concentration range. In a pharmacogenetic clinical trial there may be an analysis of the effect of a variance or variances on Phase I safety or surrogate efficacy parameters. At the same time, pharmacokinetic parameters (e.g., adsorption, distribution, metabolism, and excretion) may be a secondary objective. In a pharmacogenetic clinical study, there may be additional analysis of the gene or genes and allelic variance or variances that are suspected to be involved in these pharmacokinetic parameters. As clinical development stages progress, trial objectives focus on the appropriate dose to elicit a therapeutically relevant response. In a pharmacogenetic clinical trial, the dose or doses selected may be different than those identified based upon preclinical safety and efficacy determinations. For example, phenotypic effects of an allele depends on its frequency and also its interaction with the environment, as described earlier. Therefore, once the frequency of an allele or haplotype has been established for selected human subjects or patients, the effect of the variance on the drug responses by performing both in vitro or in vivo analyses under controlled conditions. Under these conditions, drug dosage could be adjusted accordingly. In some instances, the chosen dose may be one that is sub-optimal or is significantly less toxic so that determination of the effect of allelic variance or variances for a given treatment or human volunteer population may be appropriately tested and analyzed. In other instances, the dose may be similar to or the same as that chosen based upon *in vitro*

or *in vivo* data. In yet other instances, the dose may be greater than optimal because allelic differences or haplotypes may result in enhanced elimination, metabolic inactivation, or excretion.

Lastly, the objectives in the latter stages of clinical development center on the effect of the therapeutic intervention on the general population. In these trials, the numbers of individuals required for enrollment and the number of treatment conditions required to achieve the objectives of the trial is dictated by statistical power analysis. The number of patients required for a given pharmacogenetic clinical trial will be determined on the prior knowledge of but not exclusively limited to variance or haplotype frequency, actual disease, disorder, or condition causing allele or allele associated with the disease, disorder, or condition and their linkage relationships. For a large scale pharmacogenetic clinical study, the identified sample size will require an adequate analysis of the frequency of the allelic variance or variances within a given population, as described, for example, by Tu & Whitkemore (1999) and references therein.

Clinical trials can be designed to obscure the human subjects and/or the study coordinators from biasing that may occur during the testing of a candidate therapeutic invention. Often the candidate therapeutic intervention is compared to best medical treatment, or a placebo (a compound, agent, device, or procedure that appears identical to the candidate therapeutic intervention but is innocuous to the receiving subject). Thus, control with placebo limits efficacy perception by influencing factors such as prejudice on the part of the study participant or investigator, spontaneous alterations or variations that occur during treatment and are related to the disease studied, or are unrelated to the candidate therapeutic intervention. In pharmacogenetic clinical studies, a placebo arm or best medical therapy may be required in order to ascertain the effect of the allelic variance or variances on the efficacy or toxicology of the candidate therapeutic intervention.

Blinding refers to the lack of knowledge of the identity of the trial treatment and thus can be used to ascertain the real and not perceived effects of the candidate therapeutic intervention. Patients, trial subjects, investigators, data review committees, ancillary personnel, statisticians, and clinical trial monitors may be blinded or unblinded during the trial period. Open label trials refer to those that are unblinded; single blind is when the patient is kept unaware of the treatment groups; double blind is when both the patient and the investigator is kept unaware of the treatment groups; or a combination of these may be instituted during the trial period. Pharmacogenetic clinical trial design may include one or a combination of open label, single blind, or double blind clinical trial design because reduction of inherent biases due to the knowledge of the type of treatment the human subject or the patient is to receive will ensure detection of the accuracy of the benefits of the stratification based upon allelic variance or variances or haplotypes.

In the designed studies in all four phases, termination endpoints for trials including or excluding pharmacogenetic objectives are defined and include observation of adverse clinical events, voluntary lack of study participation either in the form of lack of adherence to the clinical protocol or sudden change in lifestyle of the participant, lack of adherence on the part of trial investigators to follow the trial protocol, death, or lack of efficacy or positive response within the test group.

Phase I of clinical development is a safety study performed in a limited (< 15) number of normal, healthy volunteers usually at single institutions. The primary endpoints in these studies is to determine pharmacokinetic parameters (i.e. adsorption, distribution, and bioavailability), dose-related side effects that are either desirable or undesirable, and metabolites that corroborate preclinical animal studies. In a Phase I pharmacogenetic clinical trial, stratification based upon allelic variance or variances of a suspected gene or genes involving any or all of the pharmacokinetic parameters will be considered and incorporated in the objectives of the trial design.

In some cases, a pharmacogenetic Phase I study may enroll healthy human volunteers and stratify these individuals based upon their genotype. In this case, a study objective may include observation of the effect of the allele/haplotype (detectable or undetectable) which the candidate therapeutic intervention may exhibit within the allelic variance, allelic variances, or haplotype groupings which can be assessed in the absence of a disease, disorder, or condition.

In some cases (e.g. cancer or medically intractable, life threatening, for those in which no medical alternative exists, or seriously debilitating diseases, disorders, or conditions) Phase I studies can include a limited number of patients with a diagnosed disease, disorder, or condition for whom clinical parameters satisfy a specified inclusion criteria (see below). These safety/limited efficacy studies can be conducted at multiple institutions to ensure enrollment of these patients. In a pharmacogenetic Phase I study that will include patients to some degree, the gene or genes and allelic variance or variances suspected to be involved in the efficacy of the candidate therapeutic intervention will be considered in the design of the inclusion criteria, the objectives, and the primary endpoints.

Phase II studies include a limited number of patients (<100) that satisfy the required inclusion criteria and do not satisfy any of the exclusion criteria of the trial design. Phase II studies can be conducted at single or multiple institutions. Inclusion criteria for patient enrollment to a clinical trial is a list of qualities for a given patient population that includes pathophysiologic clinical parameters for a given disease, disorder, or condition that can be determined by clinical diagnosis or laboratory or diagnostic test; age; gender; fertility state (e.g. pre- or postmenopausal women); coexisting medical therapies; or psychological, emotional, or cognitive state. Inclusion criteria can also include defined psychological,

emotional, or socioeconomic support by family or friends. Exclusion criteria for patient enrollment generally includes the listing of co-morbidities that may interfere with the observations of the medical or laboratory pathophysiological clinical parameters of the disease, disorder, or condition, age, gender, fertility state (e.g. pre- or postmenopausal women), or previous or concurrent medical, surgical, or diagnostic therapies. In Phase II, the primary endpoint of the study is generally limited efficacy and corroboration of the Phase I safety data in the specified patient population defined by the inclusion/exclusion criteria of the clinical protocol. Primary efficacy endpoints include observed improvements of pathophysiologic parameters that are determined medically, diagnostically (e.g. clinical laboratory values), or by surrogate measurements of the pathological state of the disease, disorder, or condition. Primary endpoints may also include limitation of pharmacologic therapies, reduction of time to death, or reduction in the progression of the disease, disorder, or condition. Surrogate markers are pathophysiologic parameters determined by medical or clinical laboratory diagnosis that are associated and have been correlated with the prognosis, progression, predisposition, or risk analysis with a disease, disorder, or condition that are not directly related to the primary diagnosed pathophysiologic condition, e.g. lowering blood pressure and coronary heart disease. Secondary endpoints are those that supplement the primary endpoint and can be used to support further clinical studies. For example, secondary endpoints include reduction in pharmacologic therapy, reduction in requirement of a medical device, or alteration of the progression of the disease disorder, or condition. Typically, in Phase II, treatment groups with varying doses are included in the study to identify the appropriate dosage and pharmacokinetic parameters to achieve maximum efficacy.

In a pharmacogenetic Phase II clinical trial, retrospective or prospective design will include the stratification of the patients based upon suspected gene or genes and allelic variance or variances involved in the pathway for pharmacodynamic or pharmacokinetic response demonstrated in the treatment groups of the candidate therapeutic intervention. These pharmacodynamic parameters may include surrogate endpoints, efficacy endpoints, or pathophysiologic thresholds. Pharmacokinetic parameters may include but are not exclusive of dosage, toxicological variables, metabolism, or excretion. Other parameters that may effect the outcome of a pharmacogenetic clinical trial may include gender, race, ethnic origins (population history), and combination of allelic variances of genes from multiple pathways, leading to but not exclusively efficacy or toxicology.

Phase III studies include multi-site, large, statistically significant, numbers of patients (<5,000) that fulfill the inclusion criteria for the study. The design of this type of trial includes power analysis to ensure the data will support the study objectives. In this large scale efficacy study, the primary endpoint is preferably defined as enhanced efficacy as

compared to placebo or best medical care for said disease, disorder, or condition. The primary endpoint may include reduction of condition progression, improvement of a specific subset of symptoms, or in requirement or perceived need of medical therapy. In a pharmacogenetic Phase III clinical study, the endpoints will be the determination of the efficacy or toxicological differences that can be demonstrated to be dependent on the stratification based upon allelic variance or variances in a gene or genes that are suspected to be involved in the efficacy or toxicological population phenotype. Further in the Phase III pharmacogenetic clinical trial, the analysis of the impact of the allelic variance or variances will be broadened from the confirmatory Phase II pharmacogenetic clinical trial data that supports the notion that the phenotypic response differences can be identified as dependent on the allelic variance or variances of a gene or genes suspected to be involved in the efficacy or toxicological response.

After the completion of a Phase III study, the data and information from all of the trials are compiled into a New Drug Application for review by the US FDA for marketing approval in the US and its territories. The NDA includes the raw (unanalyzed) clinical data, i.e. the primary endpoints or secondary endpoints, a statistical analysis of all of the included data, a document describing in detail any adverse or observed side effects, tabulation of the participant drop-outs and detailed reasons for the termination, and other specific data or details of ongoing *in vitro* or *in vivo* studies since the submission of the IND. If pharmacoeconomic objectives are a part of the clinical trial design data supporting cost or economic analyses are included in the NDA. In a pharmacogenetic clinical study, the pharmacoeconomic analyses may include demonstration or lack of benefit of the candidate therapeutic intervention in a cost benefit analysis, cost of illness study, cost minimization study, or cost utility analysis. In one or a combination of these studies, the effect of a diagnostic identification of the population and subsequent stratification based upon allelic variance or variances or haplotype of a suspected gene or genes involved in the efficacy or toxicological responses of the candidate therapeutic intervention will be used to support application for the approval for the marketing and sale of the candidate therapeutic intervention.

Phase IV studies occur after the therapeutic intervention has been approved for marketing. In these studies, retrospective data and data from a large patient population that do not necessarily fulfill the pathophysiologic requirements of the approved indication are included. In a Phase IV pharmacogenetic clinical trial, both retrospective and prospective design can be incorporated. In both cases, stratification based upon allelic variance or variances with adequate sample size in order to determine the statistical relevance of an outcome difference among the treatment groups.

Although the above listed phases of clinical development are well-established, there

are cases whereby strict Phase I, II, III development does not occur, i.e. the clinical development of candidate therapeutic interventions for serious debilitating or life threatening diseases, or for those cases whereby no medical therapeutic alternative exists. In the cases whereby the target indication for cancer or medically intractable, life threatening or seriously debilitating diseases, disorders, or conditions the US FDA has regulatory procedural mechanisms that can expedite the availability of the therapeutic intervention for patients that fall into one or more of these categories. Such development incentives include Treatment IND, Fast-Track or Accelerated review, and Orphan Drug Status. In a pharmacogenetic clinical development program for candidate therapeutic interventions for this class of indications, consideration of sample size for adequate determination of the effect allelic variance or variances may have on the outcome response or endpoints is incorporated. Further consideration may include but is not limited to accrual rate for candidate patients, and number of institutions or clinical sites required to achieve an appropriate sample size.

In additional cases of diseases, disorders, or conditions where there are no therapeutic alternatives development, sponsors may choose to expedite the development of the candidate therapeutic intervention without making use of the above FDA regulatory clinical development incentives. In these cases, the sponsor proposes expedited clinical development of a candidate therapeutic intervention due to outstanding positive or unequivocal preclinical safety and/or efficacy data.

B. Phase I Clinical Trials

Phase I clinical trials are generally designed primarily to establish a safe dose and schedule of administration for a new compound. At the same time, Phase I is the first opportunity to study the clinical pharmacology of a new compound in man. Relevant studies may include aspects of pharmacokinetic behavior, side effects and toxicity. In addition to these well established purposes, Phase I trials are increasingly being used to gather information relevant to early assessment of efficacy. Such information can be useful in making an early yes/no decision about the further development of a compound, or a family of related compounds, all being tested simultaneously in Phase I trials. Since Phase I trials are typically conducted in normal volunteers (compounds for cancer and some other terminal diseases are an exception), surrogate markers of drug effect are measured, rather than disease response. The development of sophisticated surrogate markers of pharmacodynamic effects has allowed more information on efficacy to be gathered in Phase I, and this trend will almost certainly continue as basic understanding of disease pathophysiology increases, and as more products are developed for disease prophylaxis.

Phase I studies are typically performed on a small number (<60) of healthy volunteers. Consequently, Phase I studies *as currently designed* are not amenable to

genetic analysis: the number of subjects is simply too small to detect, with adequate statistical certainty, any genetic effects on drug response that are short of all or none in magnitude. In fact, no genetic analyses of Phase I studies have been published or described in public meetings.

As described in detail elsewhere in this application, it is highly desirable to gather the information necessary to make informed decisions about clinical development as early as possible in the development process, particularly once human testing has begun and costs therefore mount quickly. Timely information may allow a drug to be killed early, or may result in an accelerated program of clinical trials. In addition to information about efficacy and safety, it is useful to have information about the existence and magnitude of genetic effects on efficacy and toxicity at the earliest possible stage. If properly managed, genetically determined heterogeneity in drug response may not be an obstacle to development. On the contrary, it may provide the basis for identification of a patient population in whom both high efficacy and safety can be achieved. Clear delineation of such a population can facilitate smaller, more targeted trials and more rapid clinical development. Consequently, the early identification of genetic determinants of drug response will, in the future, increasingly become a priority of clinical development.

Phase I trials are not necessarily confined to the initial stages of human clinical development. It is not unusual for Phase I trials to be initiated at a later stage of clinical development in order to, for example, clarify basic questions about clinical pharmacology that have arisen as a result of Phase II study data. It may be that the most efficient way to advance the genetic understanding of pharmacological responses to a compound in Phase II is to perform a Phase I trial using a specific genetic design, as described below.

2. Phase I Trials Designed for Genetic Analysis

In this invention we describe two exemplary novel methods for organization of Phase I trials that will facilitate identification and measurement of the genetic component of variation in treatment response using modest numbers of subjects. We describe how these methods can be practiced by selectively enrolling subjects who share genetic characteristics, either as a result of a familial relationship or as a result of genetic homogeneity at candidate loci believed to affect response to the candidate treatment. We show how the analysis of such individuals substantially increases the power of genetic analysis compared to analysis of unrelated individuals. We also describe methods for operating a Phase I unit capable of carrying out the novel genetic analyses

The two types of Pharmacogenetic Phase I Units described in this application will be referred to as the *Pharmacogenetic Phase I Relatives Unit* and the *Pharmacogenetic*

Phase I Outliers Unit , or the Relatives Unit and the Outliers Unit for short. The term Pharmacogenetic Phase I Unit will be used to refer to both types of Phase I Unit. The Relatives Unit requires a population comprised of groups of related individuals. The related individuals may be parents and offspring, groups of sibs, or of cousins, or any mixture of these or other groups of related individuals. The Outliers Unit requires the initial enrollment of a large number of unrelated volunteers (at least several hundreds of subjects, preferably at least one thousand, more preferably at least five thousand, and most preferably ten thousand or more individuals) willing to provide DNA for genotyping on an as-needed basis (many of these volunteers will never participate in a trial). Subsequently, small numbers of individuals are drawn from this large population for specific clinical trials, based on their genetic homogeneity at candidate loci believed likely to account for intersubject variation in response to the candidate compound.

The concept underlying these two types of Pharmacogenetic Phase I Units is similar: the idea is to recruit multiple small groups of subjects who are genetically more homogeneous than would be possible with standard nongenetic recruitment criteria. If there is a genetic component to treatment response then there should be more intragroup homogeneity and more intergroup heterogeneity in drug response measures (e.g. surrogate measures of drug response) than would be expected by chance, and there should be statistically significant differences in drug response measures between the different groups. The magnitude of such differences can provide an estimate of the magnitude of the genetic component of intersubject variation in drug response.

3. Pharmacogenetic Phase I Relatives Unit

In the Pharmacogenetic Phase I Relatives Unit, one is comparing groups of related individuals to each other and to other groups of related individuals. The underlying assumption is that one can assess the magnitude of the genetic component of variation in drug response (if any) by comparing drug response traits in related individuals with those of unrelated individuals. Two types of effect would suggest the presence of a genetic component to variation in drug response measures. First, the distribution of drug responses in related individuals may be different from that observed in the entire group, or in a group comprised of unrelated individuals. For example, a statistically significant narrowing of the distribution (e.g. smaller standard deviation in groups of related individuals compared to unrelated individuals) would indicate that individuals who share alleles are more similar to each other than individuals who do not share (as many) alleles, implying that the drug response trait is partially affected by a heritable factor or factors. Second, the mean value of the drug response measure (whether blood pressure or a cognitive test) may vary

between groups of related individuals, indicating that different alleles at loci relevant to drug response are present in the different families. (Note that the relevant trait is not blood pressure or cognition, but the response of blood pressure or cognition to a pharmacological intervention.)

Individuals can be related in any of several ways, most preferably as parent and child or as siblings. Parent - child pairs, in particular, enable one to use simple statistical techniques (e.g., regression) in order to assess the degree to which response to surrogate markers is influenced by genetic differences among individuals. However, parent-child pairs may be less suitable for some surrogate markers, especially those related to candidate drugs used to treat age-related disorders. In such a context, one can readily use clusters of siblings and/or cousins, uncle/nephew pairs or other groups of related individuals to assess the degree of genetic determination of response to a surrogate marker.

An attractive aspect of the Pharmacogenetic Phase I Relatives Unit (unlike the Outliers Unit) is that it does not require any laboratory tests to implement. One infers the degree of gene sharing between individuals from their relationship to each other. A parent is 50% genetically identical to each of his or her children; sibs are 50% genetically identical to each other on average; uncles/aunts are 25% identical to nieces/nephews on average, and so forth. Thus the degree to which two related individuals are expected to be similar as a result of genetic factors is known. Therefore no tests to determine genetic status are required (i.e. no genotyping); in fact, no knowledge of the relevant candidate loci is required at all (albeit knowledge of the relevant genes is required to develop a useful genetic diagnostic test at a later stage). Thus, the Relatives Unit provides a clear picture of the importance of heredity factors in determining drug response, regardless of our understanding of the mechanism of action of the drug, or any other aspect of drug pharmacology.

The rationale is as follows: if a surrogate drug response trait (i.e., a surrogate marker of pharmacodynamic effect that can be measured in normal subjects) is under genetic control, then related individuals, such as sibs (who share 50% of their alleles at autosomal loci on average), should have more similar responses than unrelated individuals, who share a much smaller fraction of alleles. In other words, individuals who share more alleles at the loci that affect drug response should be more similar to each other than individuals who, on average, share fewer alleles. By using statistical methods known in the art the distribution of traits of related individuals can be compared to the degree of variation in a set of unrelated individuals. The potential for insight from this kind of analysis is reflected in the fact that twin studies (in which traits of identical twins are compared to those of fraternal twins) indicate that differences among individuals in pharmacokinetic variables (e.g. compound half life, peak concentration) can be strongly

genetically determined. (For a summary of such pharmacokinetic studies, see Propping, P. [1978] Pharmacogenetics. *Rev. Physiol. Biochem. Pharmacol.* 83: 123-173.) Such studies are important because they clearly reveal genetic determination of pharmacogenetic traits (although they may overestimate its degree; see Falconer, D.S. and Mackay, T. [1996] Introduction to Quantitative Genetics, Addison Wesley Longman Ltd.).

The type of study proposed here, whether it involves comparison of parents and offspring, groups of sibs, or other groups of relatives, will also reveal the extent of genetic determination, and without requiring twins. This is a two-fold advantage; pairs of twins are more difficult to obtain than parent-child or sib-sib pairs, and one avoids the uncertainty about the genetic inferences gained from twin analysis .

Drug responses among related and unrelated individuals may be continuously or discretely distributed. In the former case, it is likely that many loci have some effect on the trait, while in the latter case, variation could be attributable to Mendelian segregation of alleles in a family (or families) with, for example, AA homozygotes giving one phenotype and Aa heterozygotes and aa homozygotes giving a second phenotype, all in the context of a relatively homogeneous genetic background.

There is a wealth of analytical techniques known in the art that can be used to assess the mode of inheritance for a particular trait and to determine the degree to which differences among individuals are genetically determined. These techniques include cluster analysis and discriminant analysis used to define traits with variable expression and the fitting of a variety of genetic models to the data, including generalized single-locus models, mixed models in which a trait is determined by a major locus and by many minor loci, and a so-called polygenic model in which many loci contribute variation to the trait, the result being a continuously-distributed phenotype (For further details, see Eaves, L.J. [1977] Inferring the causes of human variation, Journal of the Royal Statistical Society A 140: 324 -355 and Cloninger, C.R. [1988] Complex Human Traits. Pp. 312-317 in: Proceedings of the Second International Conference on Quantitative Genetics, eds., B. S. Weir, E. J. Eisen, M. M. Goodman, and G. Namkoong, Sinauer Associates, Inc). Specific statistical techniques involved in the fitting and analysis of these genetic models are also well known in the art; they include parametric and nonparametric correlation, regression, and one-way and two-way analysis of variance (For further details, see Mather, K. and Jinks, J. L. [1977] Introduction to Biometrical Genetics, Cornell University Press and Falconer, D. S. and Mackay, T. [1996] Introduction to Quantitative Genetics, Addison Wesley Longman Ltd.)

Many, perhaps most, traits of pharmacogenetic interest will be continuously-distributed. In this context, the central statistical comparison is one between the differences among average traits of different families (say, groups of sibs), or among all the members of several such families, as compared to the differences among traits within families

(among sibs). If such differences in so-called mean squares are large enough (as compared to the differences expected under the null hypothesis of no family differences), one can infer that there is a genetic component to differences among families.

Standard theory known in the art indicates that there is an inverse relationship between study size and the ability to detect a given genetic effect. So, for example, assume that the 50% of the variation among individuals is due to genetic differences. A Phase 1 trial composed of sixty individuals consisting of thirty parent-child pairs may or may not allow one to detect such a genetic effect, given the standard criterion for statistical significance ($P < 0.05$), depending on assumptions one makes about the number of loci that have major effects. However, a trial composed of 120 individuals consisting of sixty parent-child pairs would likely be sufficient to provide statistically significant evidence for a 50% heritable drug response effect. Once one parent-child pair is recruited, it is generally advantageous statistically to add additional parent-child combinations as opposed to adding additional children for a given parent.

If 75% or more of the variation in drug response among individuals is due to genetic differences, a Phase 1 trial composed of sixty individuals consisting of thirty parent-child pairs would allow one to detect such a genetic effect, given the standard criterion for statistical significance ($P < 0.05$).

Similar calculations can be made if one analyzes siblings in a Phase I trial, instead of using parent-child pairs. These calculations indicate that the more powerful approach for a Relatives Unit is generally to focus on parent-child pairs as opposed to the use of groups of siblings, especially if minimizing the number of subjects is an objective of the study. However, the use of groups of siblings may be necessary or preferable, especially if the trait in question is manifested only at a specific age. In such a case, one can readily use standard theory to compare alternative designs for the study. The overall point is that the statistical framework associated with the Relatives Unit will allow one to choose the approach that is best-suited for a given trait.

In general, techniques for measuring whether pharmacodynamic traits are under genetic control using surrogate markers of drug efficacy will be useful in obtaining an early assessment of the extent of genetically determined variation in drug response for a given therapeutic compound. Such information provides an informed basis for either stopping development at the earliest possible stage or, preferably, continuing development, but with a plan to identify and control for genetic variation so as to allow rapid progression through the regulatory approval process.

For example, it is well known that clinical trials to assess the efficacy of candidate drugs for Alzheimer's disease are long and expensive, and most such drugs are only effective in a fraction of patients. Using surrogate measures of response in normals drawn

from a population of related individuals might help to assess the contribution of genetic variation to variation in treatment response. For an acetylcholinesterase inhibitor, relevant surrogate pharmacodynamic measures might include testing erythrocyte membrane acetylcholinesterase levels in drug treated normal subjects, or testing performance on a psychometric test of short term memory, or other measures that are affected by treatment (and ideally that correlate with clinical efficacy).

Similarly, antidepressant drugs can produce a variety of effects on mood in normal subjects. Careful measurement and statistical analysis of such responses in related and unrelated normal subjects could provide an early indication of whether there is a genetic component to drug response (and hence clinical efficacy). The observation of significant variation among families would provide evidence of a pharmacogenetic effect and justify the substantial expenditure necessary for a full pharmacogenetic drug development program. Conversely, the absence of any significant familial influence on drug response in a Pharmacogenetics Relatives Unit could provide an early termination point for pharmacogenetic studies.

Again, the proposed studies do not require any knowledge of candidate loci, nor is DNA collection or genotyping required. One needs only a reliable surrogate pharmacodynamic assay and groups of related normal individuals. Standard statistical methods should permit the magnitude of the pharmacogenetic effect to be estimated. It should be a criteria for deciding whether to proceed with more intensive, gene-focused pharmacogenetic analysis during later stages of development.

4. Pharmacogenetic Phase I Outliers Unit

The prerequisites for a Pharmacogenetic Phase I Outliers Unit, as well as the type of information that can be obtained, differ in several respects from a Pharmacogenetic Phase I Relatives Unit. First, the Outliers Unit requires some knowledge of the molecular pharmacology of the candidate compound – enough knowledge to select at least one candidate gene. Second, the Outliers Unit provides information on the effect, if any, of known genetic variation in the candidate gene or genes on variation in the drug response measures. This is advantageous in that it sets the stage for pharmacogenetic analysis in later stages of clinical development. Third, the Outliers Unit does not require recruitment of relatives. Instead, one initially recruits a large population of individuals from which small subsets are drawn as necessary for specific trials based on their genotypes.

All of the individuals in the large population are initially asked to

provide DNA samples (from blood or other readily available tissue such as buccal mucosa) which can subsequently be genotyped at candidate loci of potential relevance to a particular candidate drug of interest. Over time a database of genotypes can be assembled, potentially reducing the need for genotyping later. From this large collection of subjects one then selects a group of individuals with genotypes expected to homogeneous for the drug response trait of interest (assuming that the candidate gene(s) play a significant role in drug response). The individuals with identical (and preferably homozygous) genotypes at the candidate gene(s) might comprise a collection of the common genotypes or haplotypes, or they may include some rare genotypes/haplotypes as well. The main point is that one can recruit groups consisting of any mixture of genotypes or haplotypes in order to assess the role that variation in the candidate gene(s) may play in trait determination. In this method, then, one recruits a population for clinical genetic investigation utilizing methods in statistical genetics to optimize the size and genetic composition of the population.

The mechanics of an Outlier Unit are as follows. Several thousand subjects are enrolled in the Outlier Unit with the understanding that they provide a blood sample from which DNA is extracted and stored. Each time a new outlier study is performed their sample may be genotyped. (It will not be necessary to genotype all subjects for all trials – just enough to identify subjects with the desired genotypes or haplotypes. Subjects may be paid a fee for each genotyping analysis done on their sample, regardless of whether the sample is used.) Only rarely will a particular subject have a genotype that meets the criteria for a specific outlier study (see below). When a match occurs, that subject will be invited to participate in that study. The genotyping done to identify subjects for a study will be determined by the candidate genes deemed relevant to pharmacology of the candidate drug, and by the polymorphisms or haplotypes in those candidate genes. Ideally DNA samples from several thousand subjects will be arrayed in 96 or 384 well plates so that the genotyping or haplotyping of large numbers of subjects can be performed using automated methods. Any highly accurate and inexpensive genotyping procedure will suffice, such as the methods described elsewhere in this application. Clearly it is desirable to have a stable population for genotyping, given the investment required to recruit subjects, isolate and array DNA, and accumulate a database of genotype data. Since most subjects will only rarely be invited to participate in clinical trials, the ongoing participation of subjects in the Outliers Unit must be assured by other means – for example, by a modest annual payment for remaining in the Outliers Unit, plus a fee for each occasion on which their sample is genotyped.

The power of the Outliers Unit lies in the ability to rapidly enroll individuals with virtually any desired genotype in a Phase I clinical trial. Suppose, for example, that one wants to determine the drug response phenotype of individuals homozygous for rare alleles at candidate loci. Consider a compound for which there are two loci believed likely to influence response to treatment. The first locus has alleles A and a, while the second has alleles B and b. If these loci do in fact contribute significantly to treatment response then homozygotes would be expected to exhibit the most extreme responses (assuming a dominant or codominant model). One could also measure epistatic (gene X gene) interactions on the presumption that drug response measures might be extreme in individuals homozygous for specific alleles of the two candidate genes. So, for example, one would perform a Phase I study consisting of measuring a surrogate drug response in individuals with genotypes AA/BB, aa/BB, AA/bb and aa/bb and then statistically comparing the distribution of a trait in each of these groups with the distribution of the same trait in the other groups and/or in the unfractionated (total) population. The statistical techniques for such comparisons are known in the art and include parametric and nonparametric analyses to detect differences in population averages, such as the t-test and the Mann-Whitney U test. If individuals of a given rare genotype do have significantly different surrogate drug responses when compared to each other, or when compared to the rest of the population, one can infer that the locus likely affects the trait.

The size requirements of the source population of individuals will depend on the range of allele frequencies to be analyzed. For example, if the allele frequencies for A and a are, say, 0.15 and 0.85, and for B and b are 0.2 and 0.8 then the frequency of AA homozygotes is expected to be 2.25% and BB homozygotes 4%. In the absence of any linkage between the loci, the frequency of AA/BB double homozygotes is expected to be $0.0225 \times 0.04 = 0.0009$ or about one subject in 1000. At least five subjects of each genotype should be recruited for the Outlier Unit, and preferably at least ten subjects. Thus, for studies of two loci in which the minor allele frequency for both loci is in the 0.15-0.20 range, the recruitment of individuals that are potential outliers for the trait under investigation (i.e., homozygotes at the candidate loci) will require at least 1,000 individuals and preferably 5,000 or more.

One of the most useful aspects of the Outlier Unit is that individuals with rare genotypes can be pharmacologically assessed in a small study. This addresses a serious limitation of conventional clinical trials with respect to the investigation of polygenic traits or the effect of rare alleles. Even conventional Phase III studies, which typically have the largest number of patients, are usually of insufficient size to address simple one-locus hypotheses about efficacy or toxicity with adequate statistical power (e.g. 80% or 90% power). The problem is that for each new allele that must be considered (e.g. five common

haplotypes at a candidate locus) the comparison groups are reduced and statistical power is diminished. It is therefore an especially challenging problem to test the effect of multiple alleles at a single locus, let alone interaction of alleles at several loci in determining drug response. The Outlier Unit provides a way to efficiently test for the effects of multiple alleles at a candidate locus (e.g. haplotypes), or to test for interactions between two or more candidate loci by allowing ready identification of groups of individuals who, on account of being homozygous at one or several loci of interest, should be outliers for the drug response traits of interest.

The information that can be gained from an Outliers Unit is of great value in designing subsequent efficacy trials, as it provides a basis for constraining the number of hypotheses to be tested. In lieu of such information, one is compelled to statistically test a variety of genetic models for a number of candidate loci. The correction for multiple testing necessitated by such uncertainty about the genetic model is frequently large enough to put statistically significant results beyond reach. On the other hand, if the phenotypic effect of each allele at a locus (or the effect of at least some alleles) is known from the Outliers Unit study, one is then able to design a Phase II or Phase III study that tests a relatively small number of genetic hypotheses, thereby considerably improving the statistical power of the genetic analysis in efficacy trials.

Consider a locus with two alleles, one with frequency 0.95 and the other 0.05, as revealed by genotyping the individuals in the large source population for the Outliers Unit. The two alleles combine to make three genotypes which are observed to differ in their response to a candidate compound of interest. There are several statistical comparisons that one can undertake in order to determine whether different alleles at this locus are associated with differences in response. One is to compare the average response of, say, individuals who are homozygous for the rare allele with the average response of individuals chosen at random from the source population. In this instance, the Outlier Unit is composed of a group of individuals with the rare genotype and an equal-sized group composed of random genotypes (including the rare genotype). (In general, equal group sizes are statistically more efficient; they are not necessary, however, which is fortunate since some alleles of interest might be so rare that finding, say, even ten individuals who are homozygous would be difficult.) A second kind of statistical comparison would be to compare equal-sized groups of the three genotypes (AA, Aa, aa), in order to determine whether the presence or absence of a particular allele has a significant effect on the drug response trait. In this instance, the Outlier Unit is preferably composed of equal-sized groups of the three genotypes.

Assume that being a homozygote for the rare allele of the locus described in the preceding paragraph causes a 15% average difference in a pharmacokinetic parameter (e.g., the area under curve of drug concentration in blood) as compared to random individuals.

Assume further that the Outliers Unit has a total of sixty individuals, including thirty individuals of the rare genotype and thirty individuals chosen at random. Finally, assume that the variance of individual responses is identical within the two groups and that it is equal to 0.1. Standard statistical theory indicates that thirty individuals per group is not adequate to statistically prove that there is a significant difference in average uptake rate between the groups ($P < 0.05$). Instead, with an increase to 108 individuals in each group, one would be able to provide statistical evidence for this effect. However, if we assume that homozygosity for an allele at the candidate locus causes a 30% difference in area under curve then the number of individuals required to provide statistical evidence for a difference between the two groups (for $P < 0.05$ and holding all other assumptions constant) is only twenty-seven. The number of individuals required to detect a 60% difference in area under curve (all other assumptions constant) is only seven. This calculation assumes that the loci in question affect only the average trait in each of the two groups and that the shapes of the trait distribution are identical in the two groups. While conclusions based upon such an assumption are biologically meaningful and statistically robust, in some circumstances there may be differences in the shape of the trait distributions associated with different genotypes. In particular, one or more classes of homozygous genotypes may have a narrower trait distribution (smaller variance) than another, or than the population as a whole. Such a difference can be accounted for in the analysis; in fact, it would be expected to reduce the number of subjects needed for the Outliers Unit trial (since the smaller variance of one distribution reduces the overlap between it and the other trait distribution[s] to which it is being compared). In fact, the assumption of identical variances in the homozygote and total groups is not necessarily the biologically most likely case: it is reasonable to expect that the variance of the trait in the genetically more homogeneous group may be less (if the locus in question in fact contributes to variation in the drug response trait). This effect would result in a smaller population being adequate to show a genetically determined component to the difference in treatment effect between the two groups.

Serious adverse effects occuring at low frequency are often detected in the later stages of drug development. In some cases such effects have a significant genetic component. To address this issue preemptively, an Outlier Unit can perform trials in which subjects are selected to represent only the *rare* alleles at one or more loci that are candidates for influencing the response to treatment. For example, variances occurring at 5% allele frequency are expected to occur in homozygous form in 0.25% of the population (0.05 x 0.05), and therefore may rarely, if ever, be encountered in early clinical development. Yet such subjects could readily be identified by genotyping the hundreds to thousands of patients enrolled in a Phase I Outliers Unit.

Alternatively, by insuring that all common genotypes are represented in an Outlier Unit study the contribution of a major candidate locus can be tested with a powerful statistical design. Consider a locus with five haplotypes, A, B, C, D and E, with frequencies 0.3, 0.25, 0.2, 0.15, and 0.05 (plus several additional alleles with frequency lower than 0.05). A comparison of groups of homozygous for each of the haplotypes – that is AA, BB, CC, DD and EE homozygotes – each group of equal size, provides a powerful design to measure the contribution of variation at the candidate locus to variation in drug response In this case, determination of sample sizes rests upon assumptions about the differences in average trait values for each haplotype. All other things being equal, detecting a difference is easiest when a subset of the haplotypes appears to be appreciably distinct from the rest. Such a situation allows one to make a reasonably principled decision to lump haplotypes so that one compares, say, one haplotype with all of the others. In such a circumstance, sample size calculations for testing a difference in average responses would be roughly similar to those described above. More generally, one can assess the overall heterogeneity of the traits associated with each haplotype (say, with a parametric or nonparametric analysis of variance) and one can also make individual comparisons between haplotypes (by using a multiple comparison procedure if the initial analysis of variance reveals significant heterogeneity) The identification of genetically determined phenotypic variation at such a locus the can reduce the likelihood of discrepant results due to genetic stratification in later trials.

In another embodiment of the invention, it would be useful to prospectively determine the status of polymorphisms at genes that are involved in the pharmacokinetic or pharmacodynamic action of many drugs. This would save genotyping the large Outliers Unit population each time a new project is initiated. Demand for genotyped groups of patients can be anticipated from pharmaceutical and biotechnology companies and contract research organizations (CROs). Genotyping might initially focus on common pharmacological targets such as estrogen receptors or other nuclear receptors, or on adrenergic receptors, serotonin receptors, dopamine receptors and other G protein coupled receptors. The pre-genotyped Outlier Unit population could be part of a package of services (along with genotyping assay development capability, high-throughput genotyping capacity and software and expertise in statistical genetics) designed to accelerate pharmacogenetic Phase I studies. Eventually, as the databank of genotypes is expanded, individuals with virtually any genotype or combination of genotypes can be called in for precisely designed physiological or toxicological studies designed to test for pharmacogenetic effects.

As noted earlier, the Pharmacogenetic Phase I Relatives Unit and the Pharmacogenetic Phase I Outlier Unit can provide useful information at almost any stage of clinical development. It is not unusual, for example, for a product in Phase II or even Phase III testing to be remanded to Phase I in order to clarify some aspect of toxicology or physiology. In this context, either or both of the Pharmacogenetic Phase I Units would be extremely useful to a drug development company, as studies in groups of related individuals (Relatives Unit) or in defined genetic subgroups drawn from a large genotyped population (Outliers Unit) would be an economical and efficient way to clarify the nature and extent of pharmacogenetic effects, if any, thereby paving the way for future rational development of the compound.

5. Surrogate Endpoints

As explained above, some of the most attractive applications of Pharmacogenetic Phase I Units depend on the availability of surrogate markers for pharmacodynamic drug action. The most useful surrogate markers are those which can be used in normal subjects in Phase I; which can be measured easily, inexpensively and accurately, and for which there is compelling data linking the surrogate marker with some clinically important aspect of disease biology, such as disease manifestations in various organ systems, disease progression, disease morbidity or mortality, or disparate other clinical indices known in the art. The utility of surrogate markers increases in proportion to the difficulty and cost of clincal development. Thus for a disease like Alzheimer's, where long trials involving many pateints are standard, the use of surrogate measures of, for example, cognitive ability, are highly desirable.

The standard endpoints of Phase I trials are also useful measures for analysis in a Pharmacogenetic Phase I Unit. For example, studies of compound adsorption, distribution, metabolism, excretion and bioavailability may be analyzed for their genetic component. Similarly, toxic responses and dose-related side effects may be analyzed by the pharmacogenetic methods of this invention.

6. Establishing and operating a Phase I Pharmacogenetic Relatives Unit

First, it should be noted that the information that can be gained from a Pharmacogenetic Phase I Unit provides for substantial cost savings in later stages of clinical development. Therefore it is to be expected that even if the cost of operating a Pharmacogenetic Phase I Unit exceeds the cost of operating a conventional Phase I Unit, the overall costs of clinical development are likely to be lower, thereby justifying the costs

of the Pharmacogenetic Phase I Unit. Nonetheless, it is clearly desirable to operate a Pharmacogenetic Phase I Unit as efficiently as possible. In order to make a Phase I unit an efficient business operation it is useful to (i) use statistical genetic methods to design studies that require the minimal number of subjects to achieve adequate statistical power (e.g. power of 80% to detect an effect at the $P<0.05$ level), in order to keep subject costs at a minimum, (ii) take measures to reduce the turnover of participating subjects, in view of the long term investment made in patient recruitment and (in the case of the Outliers Unit) genotyping. This may be accomplished by offering subjects financial or other incentives to encourage sustained participation in the Pharmacogenetic Phase I Unit. The types of incentives that would be useful differ between the two types of Phase I Units (see below). (iii) Secure rights to reuse genotype data and, ideally, phenotypic data collected during each Pharmacogenetic Phase I Unit trial, in order to create a database that over time will save costs by eliminating the need to repetitively genotype the same loci, and may eventually produce information of broad utility in clinical pharmacology research: namely a database on the heritability of phenotypic responses to various broad classes of compounds (benzodiazepines, statins, taxanes, etc.) and the major classes of genes involved. Such a database could become a product.

In order to efficiently set up a Phase I Pharmacogenetic Relatives Unit family participation can be encouraged by appropriate incentive compensation. For example, subjects with no participating family members might be paid $200 for participation in a study; two sibs participating in the same study might each be paid $300; if they could encourage another sib (or cousin) to participate the three related individuals might each be paid $350 for each study; parent – sib pairs might be paid $400 for each study, and so forth. This type of compensation would encourage subjects to recruit their relatives to participate in Phase I studies. To the extent that certain types of blood relationship are more useful for efficient genetical analysis, those types of related individuals could be compensated most highly. This type of compensation would increase the cost of studies, however the increased speed of setting up the Relatives Unit, and the increased retention of subjects, would compensate over time. The optimal location to establish a Pharmacogenetic Relatives Unit is in a city with a stable population, many large families, and a open attitudes toward modern technology. The size of a Relatives Unit need be little more than 150 subjects, though 250 would allow greater flexibility in drawing related subjects from different racial or ethnic groups (see below), and allow for more trials to be performed simultaneously. 400 - 500 subjects would be most preferable. Greater than 500 subjects would provide little benefit while increasing costs substantially.

Ideally subjects in the pharmacogenetic Phase I unit are of known ethnic/racial/ geographic background and willing to participate in Phase I studies, for pay, over a period

of years. For specific studies in a Relatives Unit subjects from one or more racial, ethnic or geographically defined group may be analyzed in order to (i) mirror the population in which Phase II or Phase III trials are to be conducted; (ii) determine if there are measurable differences in pharmacogenetic effects in different racial, ethnic or geographically defined groups; (iii) study the most homogeneous group possible in order to increase the chances of detecting a particular type of genetic effect.

Ideally consent for genotyping should be obtained at the same time that subjects are enrolled. Appropriate consent forms will be drafted and approved by an independent review board. It would be most efficient if blanket consent for genotyping any polymorphic site or sites deemed relevant to the pharmacology of any candidate drug could be obtained. However, if this somewhat broad type of consent is deemed inappropriate by the review board then consent could be somewhat narrowed by adding the qualification that any loci that are genotyped be relevant to a customer project. A third, more onerous arrangement would be obtain consent to genotype polymorphic sites in loci relevant to specific families of compounds, or to obtain consent for genotyping a specific list of genes. Another, still less desirable solution would be to obtain consent for genotyping on a project-by-project basis (for example by mailing out reply cards to all subjects for each study), after the specific polymorphic sites to be genotyped have been selected.

Another essential element of operating a Relatives Unit is having adequate quality control measures. One crucial aspect of quality control is an independent testing method to confirm the relatedness of the recruited subjects This can be accomplished by genotyping multiple (10 – 50) highly polymorphic loci, such as short tandem repeat sequences, in individuals believed to be related. By comparing the degree of genetic identity observed with that expected from the purported relation (e.g. 50% in the case of sibs) it is possible to ensure with considerable certainty that all related individuals are in fact related as they believe themselves to be. (Inconsistency between genotyping and reported relationship would be dealt with simply by not enrolling the unrelated individuals in any trials.)

As indicated above, methods for retention of subjects in a Phase I Outliers Unit preferably consist of making modest payments for continuing participation (i.e. continued permission to genotype under the limits of the consent); additional payments for genotyping analysis, whether or not it results in a request to participate in a clinical study; and, of course, generous compensation for participation in each Outliers Unit clinical study.

As used herein, "supplemental applications" are those in which a candidate therapeutic intervention is tested in a human clinical trial in order for the product to have an expanded label to include additional indications for therapeutic use. In these cases, the previous clinical studies of the therapeutic intervention, i.e. those involving the preclinical

safety and Phase I human safety studies can be used to support the testing of the particular candidate therapeutic intervention in a patient population for a different disease, disorder, or condition than that previously approved in the US. In these cases, a limited Phase II study is performed in the proposed patient population. With adequate signs of efficacy, a Phase III study is designed. All other parameters of clinical development for this category of candidate therapeutic interventions proceeds as described above for interventions first tested in human candidates.

As used herein, "outcomes" or "therapeutic outcomes" are used to describe the results and value of healthcare intervention. Outcomes can be multi-dimensional, e.g., including one or more of the following: improvement of symptoms; regression of the disease, disorder, or condition; economic outcomes of healthcare decisions.

As used herein, "pharmacoeconomics" is the analysis of a therapeutic intervention in a population of patients diagnosed with a disease, disorder, or condition that includes at least one of the following studies: cost of illness study (COI); cost benefit analysis (CBA), cost minimization analysis (CMA), or cost utility analysis (CUA), or an analysis comparing the relative costs of a therapeutic intervention with one or a group of other therapeutic interventions. In each of these studies, the cost of the treatment of a disease, disorder, or condition is compared among treatment groups. As used herein, costs are those economic variables associated with a disease, disorder, or condition fall into two broad categories: direct and indirect. Direct costs are associated with the medical and non-medical resources used as therapeutic interventions, including medical, surgical, diagnostic, pharmacologic, devices, rehabilitation, home care, nursing home care, institutional care, and prosthesis. Indirect costs are associated with loss of productivity due to the disease, disorder, or condition suffered by the patient or relatives. A third category, the tangible and intangible losses due to pain and suffering of a patient or relatives often is included in indirect cost studies.

As used herein, "health-related quality of life" is a measure of the impact of the disease, disorder, or condition on an individual's or group of patient's activities of daily living. Preferably, included in pharmacoeconomic studies is an analysis of the health-related quality of life. Standardized surveys or questionnaires for general health-related quality of life or disease, disorder, or condition specific determine the impact the disease, disorder, or condition has on an individuals day to day life activities or specific activities that are affected by a particular disease, disorder, or condition.

As used herein, the term "stratification" refers to the creation of a distinction between patients on the basis of a characteristic or characteristics of the patient. Generally, in the context of clinical trials, the distinction is used to distinguish responses or effects in different sets of patients distinguished according to the stratification parameters. For the present

invention, stratification preferably includes distinction of patient groups based on the presence or absence of particular variance or variances in one or more genes. The stratification may be performed only in the course of analysis or may be used in creation of distinct groups or in other ways.

A human clinical trial can result in data to support the utility of a gene variance or variances for the selection of optimal therapy. Clinical studies require no knowledge of the biological function of the gene containing the variance of the variances to be assessed, nor any knowledge of how the therapeutic invention to be assessed works at a biochemical level.

There are several important preclinical data sets that pose criteria to consider when designing a clinical study to assess the utility of a variance in a gene for selecting optimal therapy for a disease, disorder, or condition. Preferably, the data sets include one or a combination of at least of the following:

Mechanism of action of the therapeutic intervention-

If the candidate therapy (e.g. drug) has established mechanism of action, the target genes can be appropriately identified. *In vitro* data supporting altered physiologic activity of the variant forms of the gene in the presence of the therapy, assists the direction of the fundamental hypotheses and identifying the objectives for a human clinical trial.

Mechanism of metabolic transformation of the therapeutic intervention-

If *in vitro* or *in vivo* animal studies have demonstrated metabolic biotransformation of the therapeutic intervention, correlation of the effects of a variance or variances on the metabolic biotransformation of the therapeutic intervention can further assist the direction of the fundamental hypotheses and identification of the objectives of the human clinical study.

Effect of the variance or variances on therapeutic intervention-

The combined preclinical data sets should point to the premise of a controlled clinical trial of the the therapeutic intervention. The design of the trial will preferably incorporate the preclinical data sets to determine the primary and secondary endpoints. Preferably, these endpoints will include whether the therapeutic intervention is efficacious, efficacious with undesirable side effects, ineffective, ineffective with undesirable side effects, or ineffective with deleterious effects. Pharmacoeconomic analyses may be incorporated in order to support the efficacious intervention, efficacious with undesirable side effects cases, whereby the clinical outcome is positive, and economic analyses are required for the support of overall benefit to the patient and to society.

The strategies for designing a clinical trial to test the effect of a genotypic variance or variances on a physiological response to therapeutic intervention for drugs with known

mechanism of action, mechanism of biotransformation, and/or known physiologic response differentials correlated to genotypic variance or variances will be modified based upon the data and information from the preclinical studies and the patient symptomatic parameters unique to the target indication. However, the strategy (design) and the implementation (conduct) of the clinical study preferably consist of one or more of the following strategies.

A. Retrospective clinical trials.

In general the goal of retrospective clinical trials will be to test and refine hypotheses regarding genetic factors that are associated with drug responses. The best supported hypotheses can subsequently be tested in prospective clinical trials, and data from the prospective trials will likely comprise the main basis for an application to register the drug and predictive genetic test with the appropriate regulatory body. In some cases, however, it may become acceptable to use data from retrospective trials to support regulatory filings.

I. Clinical trials to study the effect of one gene locus on drug response

A. Stratify patients by genotype at one candidate variance in the candidate gene locus.

1. Genetic stratification of patients can be accomplished in several ways, including the following (where 'A' is the more frequent form of the variance being assessed and 'a' is the less frequent form):

(a) AA vs. aa

(b) AA vs. Aa vs. aa

(c) AA vs. (Aa + aa)

(d) (AA + Aa) vs. aa.

2. The effect of genotype on drug response phenotype may be affected by a variety of nongenetic factors. Therefore it may be beneficial to measure the effect of genetic stratification in a subgroup of the overall clinical trial population. Subgroups can be defined in a number of ways including, for example, biological, clinical, pathological or environmental criteria. For example, the predictive value of genetic stratification can be assessed in a subgroup or subgroups defined by:

a. Biological criteria:

i. gender (males vs. females)

ii. age (for example above 60 years of age). Two, three or more age groups may be useful for defining subgroups for the genetic analysis.

iii. hormonal status and reproductive history, including pre- vs. post-

menopausal status of women, or multiparous vs. nulliparous women

iv. ethnic, racial or geographic origin, or surrogate markers of ethnic, racial or geographic origin. (For a description of genetic markers that serve as surrogates of racial/thnic origin see, for example: Rannala, B. and J.L. Mountain, Detecting immigration by using multilocus genotypes. *Proc Natl Acad Sci U S A* , 94 (17): 9197-9201, 1997. Other surrogate markers could be used, including biochemical markers.)

b. Clinical criteria:

i. Disease status. There are clinical grading scales for many diseases. For example, the status of Alzheimer's Disease patients is often measured by cognitive assessment scales such as the mini-mental status exam (MMSE) or the Alzheimer's Disease Assessment Scale (ADAS), which includes a cognitive component (ADAS-COG). There are also clinical assessment scales for many other diseases, including cancer.

ii. Disease manifestations (clinical presentation).

c. Pathological criteria:

i. Histopathologic features of disease tissue, or pathological diagnosis. (For example there are many varieties of lung cancer: squamous cell carcinoma, adenocarcinoma, small cell carcinoma, bronchoalveolar carcinoma, etc., each of which may – which, in combination with genetic variation, may correlate with

ii. Pathological stage. A variety of diseases have pathological staging schemes

iii. Loss of heterozygosity (LOH)

iv. Pathology studies such as measuring levels of a marker protein

v. Laboratory studies such as hormone levels, protein levels, small molecule levels

3. Measure frequency of responders in each genetic subgroup. Subgroups may be defined in several ways.

i. more than two age groups

ii. age related status such as pre or post-menopausal

Stratify by haplotype at one candidate locus where the haplotype is made up of two variances, three variances or greater than three variances.

4. Statistical analysis of clinical trial data

There are a variety of statistical methods for measuring the difference between two or more groups in a clinical trial. One skilled in the art will recognize that different methods are suited to different data sets. In general, there is a family of methods customarily used in clinical trials, and another family of methods customarily used in genetic epidemiological studies. Methods from either family may be suitable for performing statistical analysis of pharmacogenetic clinical trial data.

a. Conventional Clinical Trial Statistics

Conventional clinical trial statistics include hypothesis testing and descriptive methods, as elaborated below. Guidance in the selection of appropriate statistical tests for a particular data set can be obtained from texts such as: Biostatistics: A Foundation for Analysis in the Health Sciences, 7th edition (Wiley Series in Probability and Mathematical Statistics, Applied Probability and statistics) by Wayne W. Daniel, John Wiley & Sons, 1998; Bayesian Methods and Ethics in a Clinical Trial Design (Wiley Series in Probability and Mathematical Statistics. Applied Probability Section) by J. B. Kadane (Editor), John Wiley & Sons, 1996;

b. Hypothesis testing statistical procedures

(1) One-sample procedures (binomial confidence interval, Wilcoxon signed rank test, permutation test with general scores, generation of exact permutational distributions)

(2) Two-sample procedures (*t*-test, Wilcoxon-Mann-Whitney test, Normal score test, Median test, Van der Waerden test, Savage test, Logrank test for censored survival data, Wilcoxon-Gehan test for censored survival data, Cochran-Armitage trend test, permutation test with general scores, generation of exact permutational distributions)

(3) R x C contingency tables (Fisher's exact test, Pearson's chi-squared test, Likelihood ratio test, Kruskal-Wallis test, Jonckheere-Terpstra test, Linear-by linear association test, McNemar's test, marginal homogeneity test for matched pairs)

(4) Stratified 2 x 2 contingency tables (test of homogeneity for odds ratio, test of unity for the common odds ratio, confidence interval for the common odds ratio)

(5) Stratified 2 x C contingency tables (all two-sample procedures listed above with stratification, confidence intervals for the odds ratios and trend, generation of exact permutational distributions)

(6) General linear models (simple regression, multiple regression, analysis of variance –ANOVA-, analysis of covariance, response-surface models, weighted regression, polynomial regression, partial correlation, multiple analysis of variance - MANOVA-, repeated measures analysis of variance).

(7) Analysis of variance and covariance with a nested (hierarchical) structure.

(8) Designs and randomized plans for nested and crossed experiments (completely randomized design for two treatment, split-splot design, hierarchical design, incomplete block design, latin square design)

(9) Nonlinear regression models

(10) Logistic regression for unstratified or stratified data, for binary or ordinal response data, using the logit link function, the normit function or the complementary log-log function.

(11) Probit, logit, ordinal logistic and gompit regression models.

(12) Fitting parametric models to failure time data that may be right-, left-, or interval-censored. Tested distributions can include extreme value, normal and logistic distributions, and, by using a log transformation, exponential, Weibull, lognormal, loglogistic and gamma distributions.

(13) Compute non-parametric estimates of survival distribution with right-censored data and compute rank tests for association of the response variable with other variables.

c. Descriptive statistical methods

- Factor analysis with rotations
- Canonical correlation
- Principal component analysis for quantitative variables.

- Principal component analysis for qualitative data.
- Hierarchical and dynamic clustering methods to create tree structure, dendrogram or phenogram.
- Simple and multiple correspondence analysis using a contingency table as input or raw categorical data.

Specific instructions and computer programs for performing the above calculations can be obtained from companies such as: SAS/STAT Software, SAS Institute Inc., Cary, NC, USA; BMDP Statistical Software, BMDP Statistical Software Inc., Los Angeles, CA, USA; SYSTAT software, SPSS Inc., Chicago, IL, USA; StatXact & LogXact, CYTEL Software Corporation, Cambridge, MA, USA.

d. Statistical Methods from Genetic Epidemiology

Genetic epidemiological methods can also be useful in carrying out statistical tests for the present invention.

Guidance in the selection of appropriate genetic statistical tests for analysis of a particular data set can be obtained from texts such as: Fundamentals of Genetic Epidemiology (Monographs in Epidemiology and Biostatistics, Vol 22) by M. J. Khoury, B. H. Cohen & T. H. Beaty, Oxford Univ Press, 1993; Methods in Genetic Epidemiology by Newton E. Morton, S. Karger Publishing, 1983; Methods in Observational Epidemiology, 2nd edition (Monographs in Epidemiology and Biostatistics, V. 26) by J. L. Kelsey (Editor), A. S. Whittemore & A. S. Evans, 1996; Clinical Trials : Design, Conduct, and Analysis (Monographs in Epidemiology and Biostatistics, Vol 8) by C. L. Meinert & S. Tonascia, 1986)

Strategy for the implementation of a clinical study in the case of a therapeutic with known mechanism of action:

1. Identify genes that encode proteins that perform functions related to drug absorption and/or, distribution, as well as genes related to the pharmacological action (pharmacodynamics) of the therapeutic intervention. Genes that encode proteins homologous to the proteins believed to carry out the above functions are also worth evaluation as they may carry out similar functions. Together the foregoing proteins constitute the candidate genes for affecting response of a patient to the therapeutic intervention.

2. Identify variances in the candidate genes. Initially, individual variances (and preferably their frequencies) will be identified by standard methods. Then, for genes

with more than one variance, the commonly occurring patterns of variances occurring on a single chromosome (i.e. the haplotypes) may also be established using both computational and experimental approaches. For example, a computational approach might include one of, but not limited to, the following two methods a) expectation maximization (E-M) algorithm (Excoffier and Slatkin, Mol. Biol. Evol. 1995) and, b) a combination of Parsimonious and E-M methods.

If we have a large population, implementation of the E-M method will be performed first.

A given phenotype or a sequence could come from several genotypes. This is particularly true if the sequence is heterozygous at a number of nucleotide positions. Therefore, it is not practical to just count the phenotypes and make a conclusion on the underlying genotype, because it may lead to ambiguities. To avoid such ambiguities, an alternative iterative method called the EM (expectation-maximization) algorithm is used to derive the expected genotypes for a given phenotype or a sequence. This method assumes that the population under consideration is in Hardy-Weinberg equilibrium.

For example, consider the *ABO* locus in a population. Supposing , there are *Na* people of type *A*, *Nb* people of type *B*, *Nab* people of type *AB*, and *No* people of type *O*. Assuming $N = Na + Nb + Nab + No$ in the random sample of people *N*, we cannot tell exactly how many of the *Na* people are homozygous for *A/A* and how many are heterozygotes for *A/O*.

In order to avoid this dilemma, we first assume that the expected number of genotypic frequencies in the population is in H-W equilibrium for any given (all) allele(s) frequency. This is followed by setting the allele frequencies and iteration *n*, and testing for its stability in a series of iterations, up to *m*. When the values of the initial allele frequencies stabilize at the end of series of iterations up to *m*, the resulting expected number of genotypes are assigned to phenotypes; for example, sequences or individuals.

The following steps are involved in the E-M algorithm:

1. Chose an allele or a haplotype in an expected class that occurs at the highest frequency
2. Use it as a base for the observed values and estimate the unobserved or the expected value
3. Use the second value as the true value and estimate the unobserved value from the second value
4. Continue this process (up to *m*) till you find values that do not change from one iteration to the next.

The final value is the maximum likelihood (highly likely) estimate of that allele or the haplotype

As indicated above, also among the number of methods which are used for the purpose of classifying DNA sequences, haplotypes or phenotypic characters are the parsimony methods. Parsimony principle maintains that the best explanation for the observed differences among sequences, phenotypes (individuals, species) etc., is provided by the smallest number of evolutionary changes. Alternatively, simpler hypotheses are preferable to explain a set of data or patterns, than more complicated ones, and that *ad hoc* hypotheses should be avoided whenever possible (Molecular Systematics, Hillis et al., 1996). These methods for inferring relationship among sequences operate by minimizing the number of evolutionary steps or mutations (changes from one sequence/character) required to explain a given set of data.

For example, supposing we want to obtain relationships among a set of sequences and construct a structure (tree/topology), we first count the minimum number of mutations that are required for explaining the observed evolutionary changes among a set of sequences. A structure (topology) is constructed based on this number. When once this number is obtained, another structure is tried. This process is continued for all reasonable number of structures. Finally, the structure that required the smallest number of mutational steps is chosen as the likely structure/evolutionary tree for the sequences studied.

If the computed frequency of the haplotypes are equal to the number of individuals in the population, then there will be a consideration of utilizing additional methods. For these cases and if there is a small population, then the number of haplotypes will be considered relative to the number of entrants. In a method that is a modification of previously published work (Clark, Mol Biol and Evol. 1990) homozygotes will be assigned one unambiguous haplotype. If there is a single site variance (mutation) at one of the chromosomes then it will have two haplotypes. As the number of variances (mutations) increase in the diploid chromosomes, each of these variances will be compared with the haplotypes of the original population. Then a frequency will be assigned to the new variance based upon the Hardy-Weinberg expected frequencies. (See text below for why haplotypes are useful and how to determine them experimentally, if necessary.)

3. Retrospectively reanalyze data from already completed clinical trials. Since the questions are new, the data can be treated as if it were a prospective trial, with

identified variances or haplotypes as stratification criteria and biological/clinical endpoints. Care should be taken to avoid studying a population in which there may be a link between drug-related genes and disease-related genes.

4. Select group of variances or haplotypes to differentiate: one control group including groups of variances with normal biological response one or a few case groups including groups of variances with significant biological impact

5. Establish phase III trials with selected variances as inclusion criteria and clinical/pharmacoeconomic endpoints. The number of patients required for adequate statistical power (approximately the same as in a usual phase III trial) will be determined from the phase II results and allele frequencies.

Strategy for the implementation of a clinical study in the case of a therapeutic intervention with known mechanism of biotransformation:

1. Identify genes that encode proteins that perform functions related to drug biotransformation or excretion, as well as genes related to the pharmacological action (pharmacodynamics) of the metabolized or biotransformed therapeutic intervention. Genes that encode proteins homologous to the proteins believed to carry out the above functions are also worth evaluation as they may carry out similar functions. Together the foregoing proteins constitute candidate genes for affecting response of a patient to the therapeutic intervention.

2. Identify variances in the candidate genes. Initially, individual variances will be identified by standard methods. Then, for genes with more than one variance, the commonly occurring patterns of variances occurring on a single chromosome (i.e. the haplotypes) may also be established. (See text below for why haplotypes are useful and how to determine them experimentally, if necessary.)

3. Retrospectively reanalyze data from already completed clinical trials. Since the questions are new, the data can be treated as if it were a prospective trial, with identified variances or haplotypes as stratification criteria and biological/clinical endpoints. Care should be taken to avoid studying a population in which there may be a link between drug-related genes and disease-related genes.

4. Select group of variances or haplotypes to differentiate: one control group including groups of variances with normal biological response one or a few case groups including groups of variances with significant biological impact.

5. Establish phase III trials with selected variances as inclusion criteria and clinical/pharmacoeconomic endpoints. The number of patients required for adequate statistical power (approximately the same as in a usual phase III trial) will be determined from the phase II results and allele frequencies.

Strategy for the implementation of a clinical study in the case of a therapeutic intervention where by the effect of the gene variance or variances on therapeutic intervention is known:

1. Retrospectively reanalyze data from already completed clinical trials. In this case, since the questions are new, the data can be treated as if it were a prospective trial, with identified variances or haplotypes as stratification criteria and biological/clinical endpoints. Care should be taken to avoid studying a population in which there may be a link between drug-related genes and disease-related genes.

2. Select group of variances or haplotypes to differentiate: one control group including groups of variances with normal biological response and one or a few case groups including groups of variances with significant biological impact.

3. Establish phase III or phase IV (post marketing) trials with selected variances as inclusion criteria and clinical/pharmacoeconomic endpoints. The number of patients required for adequate statistical power (approximately the same as in a usual phase III trial) will be determined from the phase II results and allele frequencies.

A clinical trial in which pharmacogenetic related efficacy or toxicity endpoints are included in the primary or secondary endpoints will be part of a retrospective or prospective clinical trial. In the design of these trials, the allelic differences will be identified and stratification based upon these genotypic differences among patient or subject groups will be used to ascertain the significance of the impact a genotype has on the candidate therapeutic intervention. Retrospective pharmacogenetic trials can be conducted at each of the phases of clinical development, with the assumption that sufficient data is available for the correlation of the physiologic effect of the candidate therapeutic intervention and the allelic variance or variances within the treatment population. In the case of a retrospective trial, the data collected from the trial can be re-analyzed by imposing the additional stratification on groups of patients by specific allelic variances that may exist in the treatment groups. Retrospective trials can be useful to ascertain whether a hypothesis that a specific variance has a significant effect on the efficacy or toxicity profile for a candidate therapeutic intervention.

A prospective clinical trial has the advantage that the trial can be designed to ensure the trial objectives can be met with statistical certainty. In these cases, power analysis, which includes the parameters of allelic variance frequency, number of treatment groups, and ability to detect positive outcomes can ensure that the trial objectives are met.

In designing a pharmacogenetic trial, retrospective analysis of Phase II or Phase III clinical data can indicate trial variables for which further analysis is required. For example, surrogate endpoints, pharmacokinetic parameters, dosage, efficacy endpoints, ethnic and gender differences, and toxicological parameters may result in data that would require further analysis and re-examination through the design of an additional trial. In these cases, analysis involving statistics,, genetics, clinical outcomes, and economic parameters may be considered prior to proceeding to the stage of designing any additional trials. Factors involved in the consideration of statistical significance may include Bonferroni analysis, permutation testing, with multiple testing correction resulting in a difference among the treatment groups that has occurred as a result of a chance of no greater than 20%, i.e. $p < 0.20$. Factors included in determining clinical outcomes to be relevant for additional testing may include, for example, consideration of the target indication, the trial endpoints, progression of the disease, disorder, or condition during the trial study period, biochemical or pathophysiologic relevance of the candidate therapeutic intervention, and other variables that were not included or anticipated in the initial study design or clinical protocol. Factors to be included in the economic significance in determining additional testing parameters include sample size, accrual rate, number of clinical sites or institutions required, additional or other available medical or therapeutic interventions approved for human use, and additional or other available medical or therapeutic interventions concurrently or anticipated to enter human clinical testing. Further, there may be patients within the treatment categories that present data that fall outside of the average or mean values, or there may be an indication of multiple allelic loci that are involved in the responses to the candidate therapeutic intervention. In these cases, one could propose a prospective clinical trial having an objective to determine the significance of the variable or parameter and its effect on the outcome of the parent Phase II trial. In the case of a pharmacogenetic difference, i.e. a single or multiple allelic difference, a population could be selected based upon the distribution of genotypes. The candidate therapeutic intervention could then be tested in this group of volunteers to test for efficacy or toxicity. The repeat prospective study could be a Phase I limited study in which the subjects would be healthy human volunteers, or a Phase II limited efficacy study in which patients which satisfy the inclusion criteria could be enrolled. In either case, the second, confirmatory trial could then be used to systematically ensure an adequate number of patients with appropriate phenotype is enrolled in a Phase III trial.

A placebo controlled pharmacogenetics clinical trial design will be one in which target allelic variance or variances will be identified and a diagnostic test will be performed to stratify the patients based upon presence, absence, or combination thereof

of these variances. In the Phase II or Phase III stage of clinical development, determination of a specific sample size of a prospective trial will be described to include factors such as expected differences between a placebo and treatment on the primary or secondary endpoints and a consideration of the allelic frequencies.

The design of a pharmacogenetics clinical trial will include a description of the allelic variance impact on the observed efficacy between the treatment groups. Using this type of design, the type of genetic and phenotypic relationship display of the efficacy response to a candidate therapeutic intervention will be analyzed. For example, a genotypically dominant allelic variance or variances will be those in which both heterozygotes and homozygotes will demonstrate a specific phenotypic efficacy response different from the homozygous recessive genotypic group. A pharmacogenetic approach is useful for clinicians and public health professionals to include or eliminate small groups of responders or non-responders from treatment in order to avoid unjustified side-effects. Further, adjustment of dosages when clear clinical difference between heterozygous and homozygous individuals may be beneficial for therapy with the candidate therapeutic intervention

In another example, a reccesive allelic variance or variances will be those in which only the homozygote recessive for that or those variances will demonstrate a specific phenotypic efficacy response different from the heterozygotes or homozygous dominants. An extension of these examples may include allelic variance or variances organized by haplotypes from additional gene or genes providing an explanation of clinical phenotypic outcome differences among the treatment groups. These types of clinical studies will point and address allelic variance and its role in the efficacy or toxicology pattern within the treatment population.

IV. Variance Identification and Use

A. Initial Identification of variances in genes

Selection of population size and composition

Prior to testing to identify the presence of sequence variances in a particular gene or genes, it is useful to understand how many individuals should be screened to provide confidence that most or nearly all pharmacogenetically relevant variances will be found. The answer depends on the frequencies of the phenotypes of interest and what assumptions we make about heterogeneity and magnitude of genetic effects. At the beginning we only know phenotype frequencies (e.g. responders vs. nonresponders, frequency of various side effects, etc.). As an example, the occurrence of serious 5-FU/FA toxicity - e.g. toxicity requiring hospitalization is often >10%. The occurrence of life

threatening toxicity is in the 1-3% range (Buroker et al. 1994). The occurrence of complete remissions is on the order of 2-8%. The lowest frequency phenotypes are thus on the order of ~2%. If we assume that (i) homogeneous genetic effects are responsible for half the phenotypes of interest and (ii) for the most part the extreme phenotypes represent recessive genotypes, then we need to detect alleles that will be present at ~10% frequency (.1 x .1 = .01, or 1% frequency of homozygotes) if the population is at Hardy-Weinberg equilibrium. To have a ~99% chance of identifying such alleles would require searching a population of 22 individuals (see Table 1 below). If the major phenotypes are associated with heterozygous genotypes then we need to detect alleles present at ~.5% frequency (2 x .005 x .995 = .00995, or ~1% frequency of heterozygotes). A 99% chance of detecting such alleles would require ~40 individuals (Table below). Given the heterogeneity of the North American population we cannot assume that all genotypes are present in Hardy-Weinberg proportions, therefore a substantial oversampling is done to increase the chances of detecting relevant variances: For our initial screening, usually, 62 individuals of known race/ethnicity are screened for variance. Variance detection studies can be extended to outliers for the phenotypes of interest to cover the possibility that important variances were missed in the normal population screening.

Table 1

	Number of subjects genotyped							
Allele frequencies	n=5	n=10	n=15	n=20	n=25	n=30	n=35	n=50
p=.99, q=.01	9.56 %	18.21	26.03	33.10	39.50	45.28	50.52	63.40
p=.97, q=.03	26.26	45.62	59.90	70.43	78.19	83.92	88.14	95.24
p=.95, q=.05	40.13	64.15	78.53	87.15	92.30	95.39	97.24	99.65
p=.93, q=.07	51.60	76.58	88.66	94.51	97.34	98.71	99.38	99.93
p =.9, q = .1	65.13	87.84	95.76	98.52	99.48	99.82	99.94	>99.99
p =.8 q = .2	89.26	98.84	99.88	99.99	>99.99	>99.99	>99.99	>99.99
p =.7 q = .3	97.17	99.92	99.99	>99.99	>99.99	>99.99	>99.99	>99.99

Likelihood of Detecting Polymorphism in a Population as a Function of Allele Frequency & Number of Individuals Genotyped

The table above shows the probability (expressed as percent) of detecting both alleles (i.e. detecting heterozygotes) at a biallelic locus as a function of (i) the allele frequencies and (ii) the number of individuals genotyped. The chances of detecting heterozygotes increases as the frequencies of the two alleles approach 0.5 (down a column), and as the number of individuals genotyped increases (to the right along a row). The numbers in the table are given by the formula: $1 - (p)^{2n} - (q)^{2n}$. Allele frequencies are designated p and q and the number of individuals tested is designated n. (Since humans are diploid, the number of alleles tested is twice the number of individuals, or 2n.)

While it is preferable that numbers of individuals, or independent sequence samples, are screened to identify variances in a gene, it is also very beneficial to identify variances using smaller numbers of individuals or sequence samples. For example, even a comparison between the sequences of

two samples or individuals can reveal sequence variances between them. Preferably, 5, 10, or more samples or individuals are screened.

Source of nucleic acid samples

Nucleic acid samples, for example for use in variance identification, can be obtained from a variety of sources as known to those skilled in the art, or can be obtained from genomic or cDNA sources by known methods. For example, the Coriell Cell Repository (Camden, N.J.) maintains over 6,000 human cell cultures, mostly fibroblast and lymphoblast cell lines comprising the NIGMS Human Genetic Mutant Cell Repository. A catalog (http://locus.umdnj.edu/nigms) provides racial or ethnic identifiers for many of the cell lines. 55 of the 62 cell lines to be genotyped (as indicated above) are drawn from this collection; the remainder were obtained from the Beijing Cancer Institute. The cell lines are derived from 21 Caucasians (of Northern, Central and Southern European origin), 8 Afro-Americans, 9 Hispanics or Mexicans, 8 Chinese, 12 Japanese, 1 American Indian, 1 East Indian, 1 Iranian, and 1 Korean. These cell lines (plus ~75 other lymphoblastoid lines) are currently in use by the inventors for variance detection studies.

Source of human DNA, RNA and cDNA samples

PCR based screening for DNA polymorphism can be carried out using either genomic DNA or cDNA produced from mRNA. For many genes, only cDNA sequences have been published, therefore the analysis of those genes is, at least initially, at the cDNA level since the determination of intron-exon boundaries and the isolation of flanking sequences is a laborious process. However, screening genomic DNA has the advantage that variances can be identified in promoter, intron and flanking regions. Such variances may be biologically relevant. Therefore preferably, when variance analysis of patients with outlier responses is performed, analysis of selected loci at the genomic level is also performed. Such analysis would be contingent on the availability of a genomic sequence or intron-exon boundary sequences, and would also depend on the anticipated biological importance of the gene in connection with the particular response.

When cDNA is to be analyzed it is very beneficial to establish a tissue source in which the genes of interest are expressed at sufficient levels that cDNA can be readily produced by RT-PCR. Preliminary PCR optimization efforts for 19 of the 29 genes in Table 2 reveal that all 19 can be amplified from

lymphoblastoid cell mRNA. The 7 untested genes belong on the same pathways and are expected to also be PCR amplifiable.

PCR Optimization

Primers for amplifying a particular sequence can be designed by methods known to those skilled in the art, including by the use of computer programs such as the PRIMER software available from Whitehead Institute/MIT Genome Center. In some cases it is preferable to optimize the amplification process according to parameters and methods known to those skilled in the art; optimization of PCR reactions based on a limited array of temperature, buffer and primer concentration conditions is utilized. New primers are obtained if optimization fails with a particular primer set.

Variance detection using T4 endonuclease VII mismatch cleavage method

Any of a variety of different methods for detecting variances in a particular gene can be utilized, such as those described in the patents and applications cited in section A above. An exemplary method is a T4 EndoVII method. The enzyme T4 endonuclease VII (T4E7) is derived from the bacteriophage T4. T4E7 specifically cleaves heteroduplex DNA containing single base mismatches, deletions or insertions. The site of cleavage is 1 to 6 nucleotides 3' of the mismatch. This activity has been exploited to develop a general method for detecting DNA sequence variances (Youil et al. 1995; Mashal and Sklar, 1995). A quality controlled T4E7 variance detection procedure based on the T4E7 patent of R.G.H. Cotton and co-workers. (Del Tito et al., in press) is preferably utilized. T4E7 has the advantages of being rapid, inexpensive, sensitive and selective. Further, since the enzyme pinpoints the site of sequence variation, sequencing effort can be confined to a 25 -30 nucleotide segment.

The major steps in identifying sequence variations in candidate genes using T4E7 are: (1) PCR amplify 400-600 bp segments from a panel of DNA samples; (2) mix a fluorescently-labeled probe DNA with the sample DNA; (3) heat and cool the samples to allow the formation of heteroduplexes; (4) add T4E7 enzyme to the samples and incubate for 30 minutes at 37^{o}C, during which cleavage occurs at sequence variance mismatches; (5) run the samples on an ABI 377 sequencing apparatus to identify cleavage bands, which indicate the presence and location of variances in the sequence; (6) a subset of PCR

fragments showing cleavage are sequenced to identify the exact location and identity of each variance.

The T4E7 Variance Imaging procedure has been used to screen particular genes. The efficiency of the T4E7 enzyme to recognize and cleave at all mismatches has been tested and reported in the literature. One group reported detection of 81 of 81 known mutations (Youil et al. 1995) while another group reported detection of 16 of 17 known mutations (Mashal and Sklar, 1995). Thus, the T4E7 method provides highly efficient variance detection.

DNA sequencing

A subset of the samples containing each unique T4E7 cleavage site is selected for sequencing. DNA sequencing can , for example, be performed on ABI 377 automated DNA sequencers using BigDye chemistry and cycle sequencing. Analysis of the sequencing runs will be limited to the 30-40 bases pinpointed by the T4E7 procedure as containing the variance. This provides the rapid identification of the altered base or bases.

In some cases, the presence of variances can be inferred from published articles which describe Restriction Fragment Length Polymorphisms (RFLP). The sequence variances or polymorphisms creating those RFLPs can be readily determined using convention techniques, for example in the following manner. If the RFLP was initially discovered by the hybridization of a cDNA, then the molecular sequence of the RFLP can be determined by restricting the cDNA probe into fragments and separately hybridizing to a Southern blot consisting of the restriction digestion with the enzyme which reveals the polymorphic site, identifying the sub-fragment which hybridizes to the polymorphic restriction fragment, obtaining a genomic clone of the gene (e.g., from commercial services such as Genome Systems (Saint Louis, Missouri) or Research Genetics (Alabama) which will provide appropriate genomic clones on receipt of appropriate primer pairs). Using the genomic clone, restrict the genomic clone with the restriction enzyme which revealed the polymorphism and isolate the fragment which contains the polymorphism, e.g., identifying by hybridization to the cDNA which detected the polymorphism. The fragment is then sequenced across the polymorphic site. A copy of the other allele can be obtained by PCT from addition samples.

Variance detection using sequence scanning

In addition to the physical methods, e.g., those described above and

others known to those skilled in the art (see, e.g., Housman, U.S. Patent 5,702,890; Housman et al., U.S. Patent Application 09/045,053), variances can be detected using computational methods, involving computer comparison of sequences from two or more different biological sources, which can be obtained in various ways, for example from public sequence databases. The term "variance scanning" refers to a process of identifying sequence variances using computer-based comparison and analysis of multiple representations of at least a portion of one or more genes. Computational variance detection involves a process to distinguish true variances from sequencing errors or other artifacts, and thus does not require perfectly accurate sequences. Such scanning can be performed in a variety of ways as known to those skilled in the art, preferably, for example, as described in Stanton and Adams, U.S. Patent Application filed April 26, 1999, 09/300,747.

While the utilization of complete cDNA sequences is highly preferred, it is also possible to utilize genomic sequences. Such analysis may be desired where the detection of variances in or near splice sites is sought. Such sequences may represent full or partial genomic DNA sequences for a gene or genes. Also, as previously indicated, partial cDNA sequences can also be utilized although this is less preferred. As described below, the variance scanning analysis can simply utilize sequence overlap regions, even from partial sequences. Also, while the present description is provided by reference to DNA, e.g., cDNA, some sequences may be provided as RNA sequences, e.g., mRNA sequences. Such RNA sequences may be converted to the corresponding DNA sequences, or the analysis may use the RNA sequences directly.

B. Determination of Presence or Absence of Known Variances

The identification of the presence of previously identified variances in cells of an individual, usually a particular patient, can be performed by a number of different techniques as indicated in the Summary above. Such methods include methods utilizing a probe which specifically recognizes the presence of a particular nucleic acid or amino acid sequence in a sample. Common types of probes include nucleic acid hybridization probes and antibodies, for example, monoclonal antibodies, which can differentially bind to nucleic acid sequences differing in one or more variance sites or to polypeptides which differ in one or more amino acid residues as a result of the nucleic acid sequence variance or variances. Generation and use of such probes is well-known in the art and so is not described in detail herein.

Preferably, however, the presence or absence of a variance is determined using nucleotide sequencing of a short sequence spanning a previously identified variance

site. This will utilize validated genotyping assays for the polymorphisms previously identified. Since both normal and tumor cell genotypes can be measured, and since tumor material will frequently only be available as paraffin embedded sections (from which RNA cannot be isolated), it will be necessary to utilize genotyping assays that will work on genomic DNA. Thus PCR reactions will be designed, optimized, and validated to accommodate the intron exon structure of each of the genes. If the gene structure has been published (as it has for some of the listed genes), PCR primers can be designed directly. However, if the gene structure is unknown, the PCR primers may need to be moved around in order to both span the variance and avoid exon-intron boundaries. In some cases one-sided PCR methods such as bubble PCR (Ausubel et al. 1997) may be useful to obtain flanking intronic DNA for sequence analysis.

Using such amplification procedures, the standard method used to genotype normal and tumor tissues will be DNA sequencing. PCR fragments encompassing the variances will be cycle sequenced on ABI 377 automated sequencers using Big Dye chemistry

C. Correlation of the Presence or Absence of Specific Variances with Differential Treatment Response

Prior to establishment of a diagnostic test for use in the selection of a treatment method or elimination of a treatment method, the presence or absence of one or more specific variances in a gene or in multiple genes is correlated with a differential treatment response. (As discussed above, usually the existence of a variable response and the correlation of such a response to a particular gene is performed first.) Such a differential response can be determined using prospective and/or retrospective data. Thus, in some cases, published reports will indicate that the course of treatment will vary depending on the presence or absence of particular variances. That information can be utilized to create a diagnostic test and/or incorporated in a treatment method as an efficacy or safety determination step.

Usually, however, the effect of one or more variances is separately determined. The determination can be performed by analyzing the presence or absence of particular variances in patients who have previously been treated with a particular treatment method, and correlating the variance presence or absence with the observed course, outcome, and/or development of adverse events in those patients. This approach is useful in cases where both the observation of treatment effects was clearly recorded and cell samples are available or can be obtained. Alternatively, the analysis can be performed prospectively, where the presence or absence of the variance or variances in an individual is determined and the course, outcome, and/or development of adverse

events in those patients is subsequently or concurrently observed and then correlated with the variance determination.

Analysis of Haplotypes Increases Power of Genetic Analysis

Usually, variation in activity due to a single gene or a single genetic variance in a single gene is not sufficient to account for observed variation in patient response to a treatment, e.g., a drug, there are often other factors that account for some of the variation in patient response. This is to be expected as drug response phenotypes usually vary continuously, and such (quantitative) traits are typically influenced by a number of genes (Falconer and Mackay, 1997). Although it is impossible to determine *a priori* the number of genes influencing a quantitative trait, often only a few loci have large effects, where a large effect is 5-20% of total variation in the phenotype (Mackay, 1995).

Having identified genetic variation in enzymes that may affect action of a specific drug, it is useful to efficiently address its relation to phenotypic variation. The sequential testing for correlation between phenotypes of interest and single nucleotide polymorphisms may be adequate to detect associations if there are major effects associated with single nucleotide changes; certainly it is useful to this type of analysis. However there is no way to know in advance whether there are major phenotypic effects associated with single nucleotide changes and, even if there are, there is no way to be sure that the salient variance has been identified by screening cDNAs. A more powerful way to address the question of genotype-phenotype correlation is to assort genotypes into haplotypes. (A haplotype is the cis arrangement of polymorphic nucleotides on a particular chromosome.) Haplotype analysis has several advantages compared to the serial analysis of individual polymorphisms at a locus with multiple polymorphic sites.

(1) Of all the possible haplotypes at a locus (2^n haplotypes are theoretically possible at a locus with n binary polymorphic sites) only a small fraction will generally occur at a significant frequency in human populations. Thus, association studies of haplotypes and phenotypes will involve testing fewer hypotheses. As a result there is a smaller probability of Type I errors, that is, false inferences that a particular variant is associated with a given phenotype.

(2) The biological effect of each variance at a locus may be different both in magnitude and direction. For example, a polymorphism in the 5' UTR may affect

translational efficiency, a coding sequence polymorphism may affect protein activity, a polymorphism in the 3' UTR may affect mRNA folding and half life, and so on. Further, there may be interactions between variances: two neighboring polymorphic amino acids in the same domain - say cys/arg at residue 29 and met/val at residue 166 - may, when combined in one sequence, for example, 29cys-166val, have a deleterious effect, whereas 29cys-166met, 29arg-166met and 29arg-166val proteins may be nearly equal in activity. Haplotype analysis is the best method for assessing the interaction of variances at a locus.

(3) Templeton and colleagues have developed powerful methods for assorting haplotypes and analyzing haplotype/phenotype associations (Templeton et al., 1987). Alleles which share common ancestry are arranged into a tree structure (cladogram) according to their time of origin in a population. Haplotypes that are evolutionarily ancient will be at the center of the branching structure and new ones (reflecting recent mutations) will be represented at the periphery, with the links representing intermediate steps in evolution. The cladogram defines which haplotype-phenotype association tests should be performed to most efficiently exploit the available degrees of freedom, focusing attention on those comparisons most likely to define functionally different haplotypes (Haviland et al., 1995). This type of analysis has been used to define interactions between heart disease and the apolipoprotein gene cluster (Haviland et al 1995) and Alzheimer's Disease and the Apo-E locus (Templeton 1995) among other studies, using populations as small as 50 to 100 individuals.

Methods for determining haplotypes

The goal of haplotyping will be to identify the common haplotypes at selected loci that have multiple sites of variance. Haplotypes will usually be determined at the cDNA level. Two general approaches to identification of haplotyes will be employed. First, haplotypes will be inferred from the pattern of allele segregation in families collected by the Centre d'Etude Polymorphisme Humaine. Cell lines from these families are available from the Coriell Repository. Cell lines for all members of families 884, 102, 104 and 1331 are currently utilized. Cell lines from six additional families will also be used to increase the likelihood of detecting common haplotypes. This approach will be useful for cataloging common haplotypes and for validating methods on samples with known haplotypes. Second, haplotypes will be determined directly from cDNA using the T4E7 procedure. T4E7 cleaves mismatched heteroduplex DNA at the site of the mismatch. If a heteroduplex contains only one mismatch, cleavage will result in the generation of two fragments. However, if a single

heteroduplex (allele) contains two mismatches, cleavage will occur at two different sites resulting in the generation of three fragments. The appearance of a fragment whose size corresponds to the distance between the two cleavage sites is diagnostic of the two mismatches being present on the same strand (allele). Thus, T4E7 can be used to determine haplotypes in diploid cells.

An alternative method, allele specific PCR, may be used for haplotyping. The utility of allele specific PCR for haplotyping has already been established (Michalatos-Beloin et al., 1996; Chang et al. 1997). Opposing PCR primers are designed to cover two sites of variance (either adjacent sites or sites spanning one or more internal variances). Two versions of each primer are synthesized, identical to each other except for the 3' terminal nucleotide. The 3' terminal nucleotide is designed so that it will hybridize to one but not the other variant base. PCR amplification is then attempted with all four possible primer combinations in separate wells. Because Taq polymerase is very inefficient at extending 3' mismatches, the only samples which will be amplified will be the ones in which the two primers are perfectly matched for sequences on the same strand (allele). The presence or absence of PCR product allows haplotyping of diploid cell lines. At most two of four possible reactions should yield products. This procedure has been successfully applied, for example, to haplotype the DPD amino acid polymorphisms.

For haplotypes identified herein, haplotypes were identified by examining genotypes from each cell line. This list of genotypes was optimized to remove variance sites/individuals with incomplete information, and the genotype from each remaining cell line was examined in turn. The number of heterozygotes in the genotype were counted, and those genotypes containing more than one heterozygote were discarded, and the rest were gathered in a list for storage and display.For haplotypes identified herein, haplotypes were identified by examining genotypes from each cell line. This list of genotypes was optimized to remove variance sites/individuals with incomplete information, and the genotype from each remaining cell line was examined in turn. The number of heterozygotes in the genotype were counted, and those genotypes containing more than one heterozygote were discarded, and the rest were gathered in a list for storage and display.

D. Selection of Treatment Method Using Variance Information

1. General

Once the presence or absence of a variance or variances in a gene or genes is shown to correlate with the efficacy or safety of a treatment method, that information can be used to select an appropriate treatment method for a particular patient. In the

case of a treatment which is more likely to be effective when administered to a patient who has at least one copy of a gene with a particular variance or variances (in some cases the correlation with effective treatment is for patients who are homozygous for variance or set of variances in a gene) than in patients with a different variance or set of variances, a method of treatment is selected (and/or a method of administration) which correlates positively with the particular variance presence or absence which provides the indication of effectiveness. As indicated in the Summary, such selection can involve a variety of different choices, and the correlation can involve a variety of different types of treatments, or choices of methods of treatment. In some cases, the selection may include choices between treatments or methods of administration where more than one method is likely to be effective, or where there is a range of expected effectiveness or different expected levels of contra-indication or deleterious effects. In such cases the selection is preferably performed to select a treatment which will be as effective or more effective than other methods, while having a comparatively low level of deleterious effects. Similarly, where the selection is between method with differing levels of deleterious effects, preferably a method is selected which has low such effects but which is expected to be effective in the patient.

Alternatively, in cases where the presence or absence of the particular variance or variances is indicative that a treatment or method of administration is more likely to be ineffective or contra-indicated in a patient with that variance or variances, then such treatment or method of administration is generally eliminated for use in that patient.

2. Diagnostic Methods

Once a correlation between the presence and absence of at least one variance in a gene or genes and an indication of the effectiveness of a treatment, the determination of the presence or absence of that at least one variance provides diagnostic methods, which can be used as indicated in the Summary above to select methods of treatment, methods of administration of a treatment, methods of selecting a patient or patients for a treatment. and others aspects in which the determination of the presence or absence of those variances provides useful information for selecting or designing or preparing methods or materials for medical use in the aspects of this invention. As previously stated, such variance determination or diagnostic methods can be performed in various ways as understood by those skilled in the art.

In certain variance determination methods, it is necessary or advantageous to amplify one or more nucleotide sequences in one or more of the genes identified herein. Such amplification can be performed by conventional methods, e.g., using polymerase chain reaction (PCR) amplification. Such amplification methods are well-known to

those skilled in the art and will not be specifically described herein. For most applications relevant to the present invention, a sequence to be amplified includes at least one variance site, which is preferably a site or sites which provide variance information indicative of the effectiveness of a method of treatment or method of administration of a treatment, or effectiveness of a second method of treatment which reduces a deleterious effect of a first treatment method, or which enhances the effectiveness of a first method of treatment. Thus, for PCR, such amplification generally utilizes primer oligonucleotides which bind to or extent through at least one such variance site under amplification conditions.

For convenient use of the amplified sequence, e.g., for sequencing, it is beneficial that the amplified sequence be of limited length, but still long enough to allow convenient and specific amplification. Thus, preferably the amplified sequence has a length as described in the Summary.

Also, in certain variance determination, it is useful to sequence one or more portions of a gene or genes, in particular, portions of the genes identified in this disclosure. As understood by persons familiar with nucleic acid sequencing. In particular, sequencing can utilize dye termination methods and mass spectrometric methods. The sequencing generally involves a nucleic acid sequence which includes a variance site as indicated above in connection with amplification. Such sequencing can directly provide determination of the presence or absence of a particular variance or set of variances, e.g., a haplotype, by inspection of the sequence (visually or by computer). Such sequencing is generally conducted on PCR amplified sequences in order to provide sufficient signal for practical or reliable sequence determination.

Likewise, in certain variance determinations, it is useful to utilize a probe or probes. As previously described, such probes can be of a variety of different types.

IV. Pharmaceutical Compositions, Including Pharmaceutical Compositions Adapted to be Preferentially Effective in Patients Having Particular Genetic Characteristics

1. General

The methods of the present invention, in many cases will utilize conventional pharmaceutical compositions, but will allow more advantageous and beneficial use of those compositions due to the ability to identify patients who are likely to benefit from a particular treatment or to identify patients for whom a particular treatment is less likely to be effective or for whom a particular treatment is likely to produce undesirable or intolerable effects. However, in some cases, it is advantageous to utilize compositions which are

adapted to be preferentially effective in patients who possess particular genetic characteristics, i.e., in whom a particular variance or variances in one or more genes is present or absent (depending on whether the presence or the absence of the variance or variances in a patient is correlated with an increased expectation of beneficial response). Thus, for example, the presence of a particular variance or variances may indicate that a patient can beneficially receive a significantly higher dosage of a drug than a patient having a different variance or variances.

2. Regulatory Indications and Restrictions

The sale and use of drugs and the use of other treatment methods usually are subject to certain restrictions by a government regulatory agency charged with ensuring the safety and efficacy of drugs and treatment methods for medical use, and approval is based on particular indications. In the present invention it is found that variability in patient response or patient tolerance of a drug or other treatment often correlates with the presence or absence of particular variances in particular genes. Thus, it is expected that such a regulatory agency may indicate that the approved indications for use of a drug with a variance-related variable response or toleration include use only in patients in whom the drug will be effective, and/or for whom the administration of the drug will not have intolerable deleterious effects, such as excessive toxicity or unacceptable side-effects. Conversely, the drug may be given for an indication that it may be used in the treatment of a particular disease or condition where the patient has at least one copy of a particular variance, variances, or variant form of a gene. Even if the approved indications are not narrowed to such groups, the regulatory agency may suggest use limited to particular groups or excluding particular groups or may state advantages of use or exclusion of such groups or may state a warning on the use of the drug in certain groups. Consistent with such suggestions and indications, such an agency may suggest or recommend the use of a diagnostic test to identify the presence or absence of the relevant variances in the prospective patient. Such diagnostic methods are described in this description. Generally, such regulatory suggestion or indication is provided in a product insert or label, and is generally reproduced in references such as the Physician's Desk Reference (PDR). Thus, this invention also includes drugs or pharmaceutical compositions which carry such a suggestion or statement of indication or warning or suggestion for a diagnostic test, and which may also be packaged with an insert or label stating the suggestion or indication or warning or suggestion for a diagnostic test.

In accord with the possible variable treatment responses, an indication or suggestion can specify that a patient be heterozygous, or alternatively, homozygous for a particular variance or variances or variant form of a gene. Alternatively, an indication

or suggestion may specify that a patient have no more than one copy, or zero copies, of a particular variance, variances, or variant form of a gene.

A regulatory indication or suggestion may concern the variances or variant forms of a gene in normal cells of a patient and/or in cells involved in the disease or condition. For example, in the case of a cancer treatment, the response of the cancer cells can depend on the form of a gene remaining in cancer cells following loss of heterozygosity affecting that gene. Thus, even though normal cells of the patient may contain a form of the gene which correlates with effective treatment response, the absence of that form in cancer cells will mean that the treatment would be less likely to be effective in that patient than in another patient who retained in cancer cells the form of the gene which correlated with effective treatment response. Those skilled in the art will understand whether the variances or gene forms in normal or disease cells are most indicative of the expected treatment response, and will generally utilize a diagnostic test with respect to the appropriate cells. Such a cell type indication or suggestion may also be contained in a regulatory statement, e.g., on a label or in a product insert.

3. Preparation and Administration of Drugs and Pharmaceutical Compositions Including Pharmaceutical Compositions Adapted to be Preferentially Effective in Patients Having Particular Genetic Characteristics

A particular compound useful in this invention can be administered to a patient either by itself, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient(s). In treating a patient exhibiting a disorder of interest, a therapeutically effective amount of a agent or agents such as these is administered. A therapeutically effective dose refers to that amount of the compound that results in amelioration of one or more symptoms or a prolongation of survival in a patient.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by HPLC.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See *e.g.* Fingl et. al., in The Pharmacological Basis of Therapeutics, 1975, Ch. 1 p.1). It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administrated dose in the management of disorder of interest will vary with the severity of the condition to be treated and the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

Depending on the specific conditions being treated, such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990). Suitable routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous

injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions. The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levitating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

EXAMPLES

Example 1 Gene Identification

Metabolic Pathways that Affect 5-FU/FA Action

The biochemical pathways of 5-FU metabolism have been studied extensively. Likewise, folate metabolism has been well investigated and the enzymes that form and consume 5, 10-methylenetetrahydrofolate are well known. The principal metabolic pathways that influence the pharmacologic action of 5-FU are summarized below.

De novo and salvage routes of pyrimidine nucleotide formation (5-FU anabolism) and inhibition of thymidylate synthase

5-FU is a biologically inactive pyrimidine analog which must be phosphorylated and ribosylated to the nucleoside analog fluorodeoxyuridine monophosphate (FdUMP) to have clinical activity. FdUMP formation can occur via several routes, summarized in Figure 1. 5-FU may be converted by uridine phosphorylase to fluorouridine (FUdR; the reverse reaction is catalyzed by uridine nucleosidase) and then to fluorouridine

monophosphate (FUMP) by uridine kinase, or FUMP may be formed from 5-FU in one step via transfer of a phosphoribosyl group from 5-phosphoribosyl-1-pyrophosphate (PRPP), catalyzed by orotate phosphoribosyl transferase. FUMP can be converted to FUDP and subsequently FUTP by a nucleoside monophosphate kinase and nucleoside diphosphate kinase, respectively. FUTP is incorporated into RNA by RNA polymerases, which may account in part for 5-FU toxicity as a result of effects on processing or function (e.g. translation). Alternatively, FUDP may be reduced to the dinucleotide level, FdUDP (fluorodeoxyuridine diphosphate) by ribonucleotide diphosphate reductase, a heterodimeric enzyme. FdUDP can then be converted to FdUTP by nucleoside diphosphate kinase and incorporated into DNA by DNA polymerases which may account for some 5-FU toxicity. Fluoropyrimidine modified DNA may also be targeted by the nucleotide excision repair process. The more important path of FdUDP metabolism with respect to anticancer effects, however, is believed to be conversion to FdUMP by nucleoside diphosphatase (or cytidylate kinase, a bidirectional enzyme). dUMP is the precursor of dTMP in de novo pyrimidine biosynthesis, a reaction catalyzed by thymidylate synthase and which consumes 5,10-methylenetetrahydrofolate, producing 7,8 dihydrofolate. FdUMP, however, forms an inhibitory (probably covalent) complex with thymidylate synthase in the presence of 5,10-methylenetetrahydrofolate, thereby blocking formation of thymidylate (other than by the salvage pathway via thymidine kinase). The complex anabolism of FdUMP can be simplified by giving the deoxyribonucleoside of 5-FU, 5-fluorodeoxyuridine (also called floxuridine; FUdR), which can be converted to FdUMP in one step by thymidine kinase. However, FUdR is also rapidly converted back to 5-FU by the bidirectional enzyme thymidine phophorylase.

5-FU catabolism.

Metabolic elimination of 5-FU occurs via a three step pathway leading to • -alanine. The first and rate limiting enzyme in the elimination pathway is dihydropyrimidine dehydrogenase (DPD), which transforms more than 80% of a dose of 5-FU to the inactive dihydrofluorouracil form. Subsequently dihydropyrimidinase catalyzes opening of the pyrimidine ring to form 5-fluoro-• -ureidopropionate and then • -ureidopropionase (also called • -alanine synthase) catalyzes formation of 2-fluoro-• -alanine. The first two reactions are reversible.

The distribution of activity of these enzymes in human populations has not been established, however, a recent population survey of urinary pyrimidine levels in 1,133 adults revealed that levels of dihydrouracil range from 0 - 59 uM/g of creatinine, while uracil levels ranged from 0 - 130 uM/g creatinine (Hayashi et al., 1996), suggesting

variation in the activity of enzymes of pyrimidine metabolism. It is worth noting that in animal studies catabolites of 5-FU apparently account for some fraction of 5-FU toxicity (Davis et al., 1994; Spector et al., 1995). This result is the rationale for current human trials of 5-FU combined with DPD inhibitors: if the 5-fluoro- metabolites are responsible for toxicity, then blocking their formation by inhibition of DPD, while simultaneously decreasing 5-FU dosage to compensate for the block in catabolism and excretion, should result in a better therapeutic index.

Folinic acid conversion to tetrahydrofolate.

The conversion of FA to 5,10MTHF can occur via several routes, illustrated in Figure 2.

Intracellular reduced folate levels can potentiate 5-FU action by increasing 5,10-methylenetetrahydrofolate levels (5,10-methyleneTHF; see center of Figure 2), thereby stabilizing the ternary inhibitory complex formed with thymidylate synthase and FdUMP. This is the basis for therapeutic modulation of 5-FU with FA. As can be seen in Figure 2, conversion of folinic acid (5-formylTHF) to 5,10-methenylTHF, the precursor of 5,10-methyleneTHF, requires methenyltetrahydrofolate synthetase (enzyme 2 in the Figure). Also, levels of 5,10-methyleneTHF may be affected directly by the activity of methyleneltetrahydrofolate dehydrogenase, methyleneltetrahydrofolate reductase, serine transhydroxymethylase and the glycine cleavage system enzymes (7, 8, 10 and 11 in Fig. 2), and indirectly by the other enzymes shown in the Figure.

Cell uptake of pyrimidine nucleosides and folinic acid

Human cells have five concentrative nucleoside transporters with varying patterns of tissue distribution (see review by Wang et al., 1997). Two transporters, one with preference for purines and one for pyrimidines have been cloned recently (Felipe et al., 1998). 5-FU entry into cells may be modulated by activity of these transporters, particularly the pyrimidine transporter, although one prospective randomized clinical trial in which the nucleoside transport inhibitor dipyramidole was paired with 5-FU and FA failed to show a difference in outcome compared to 5-FU/FA alone (Kohne et al., 1995). Several folate transport systems have been identified in human cells. Folate receptor 1 (FR1) is a high affinity (nanomolar range) receptor for reduced folates. Three restriction fragment length polymorphisms (RFLPs) have been reported at the FR1 locus (Campbell et al., 1991). Reduced folates are also transported by folate receptor gamma and by a low affinity (1 uM) folate transporter. 15-fold variation in levels of folate transporter have been described in unselected tumor cell lines (Moscow et al., 1997).

Catalog allelic variation in enzymes that affect 5-FU and FA action
Select genes for analysis of sequence variation

In accord with the pathway description above, variation in either expression levels or intrinsic activity of the proteins involved in (i) cellular uptake of pyrimidines or reduced folate, (ii) conversion of 5-FU to the nucleotide form FdUMP, FUTP or FdUTP, (iii) catabolism of 5-FU, (iv) conversion of folinic acid to 5,10-methylenetetrahydrofolate or (iv) depletion of cellular 5,10-methylenetetrahydrofolate may be causally related to variation in clinical effect of 5-FU/FA. Table 3 below lists exemplary genes that will be, or already have been screened for polymorphism.

Table 2

Folate Transport	5-FU Anabolism	5-FU Catabolism	Conversion of Folinic Acid to 5,10-MethyleneTHF
Folate receptor 1 (•) GenBank M28099	Uridine phosphorylase GenBank X90858	*Dihydropyrimidine Dehydrogenase* GenBank U09178	Methylenetetrahydrofolate synthase GenBank L38298
Folate receptor (•) GenBank J02876	Thymidine phosphorylase GenBank S72487	Dihydropyrimidinase GenBank D78011	Methenyltetrahydrofolate cyclohydrolase; formyltetrahydrofolate synthetase; Methenyltetrahydrofolate dehydrogenase (one locus) GenBank J04031
Folate Transporter (SLC19A1) GenBank U19720	Orotate phosphoribosyltransferase GenBank J03626	**Inhibition of dTMP Synthesis**	*Methylenetetrahydrofolate reductase* GenBank U09806
Folate receptor (•) GenBank Z32564	Uridine Kinase GenBank D78335	*Thymidylate synthase* GenBank X02308	Serine transhydroxymethylase 1 GenBank L11931
	Thymidine kinase 1 GenBank K02581; Thymidine Kinase 2 GenBank U77088		*Methionine synthetase* GenBank U50929
Pyrimidine Transport	Ribonucleoside reductase: *M1 subunit* GenBank X59543 M2 subunit GenBank X59618	**Folate Polyglutamation**	Glycine cleavage system, Protein H:GenBank M69175; Protein P: GenBank M64590; Protein T: GenBank D13811
Nucleoside transporter 1	Nucleoside diphosphate kinase, A subunit GenBank U29200	Folylpolyglutamate synthetase GenBank M98045	*Dihydrofolate reductase* GenBank J00140
	B subunit GenBank X58965	Folylpolyglutamate hydrolase GenBank	

There are 27 genes in the above Table. Six genes which have already been surveyed for polymorphism are italicized. The following genes do not appear in the Table because there is no human cDNA in GenBank: 5-FU anabolism: Uridine monophosphate kinase; 5-FU catabolism: b-ureidopropionase; Folate metabolism: Glutamate formiminotransferase, Formiminotetrahydrofolate cyclodeaminase,

Formyltetrahydrofolate hydrolase, Formyltetrahydrofolate dehydrogenase, and Protein L of the glycine cleavage system. Other genes not listed in the Table include DNA and RNA polymerases and DNA repair enzymes, some of which (e.g. DNA polymerase b and RNA polymerase II 220 and 33 kD subunits) have already been screened for polymorphism. Those additional genes are also useful in the present invention.

For several potential candidate genes there are mammalian cDNAs in GenBank but no human cDNA. For example, there is a 1,420 nucleotide full length rat β-ureidopropionase cDNA. Four overlapping human ESTs (F06711, H19181, R11806 and W55897) span 691 nucleotides of the rat coding sequence with >90% nucleotide identity. For selected candidate genes of likely importance, such as β-ureidopropionase, polymorphism analysis will be carried out on the available human sequence from dbEST.

Example 2 Variance Identification - Variances in Genes That Can Affect 5-FU/FA Action

Exemplary genes related to modulation of the action of 5-FU/FA have been analyzed for genetic variation; thymidylate synthase, ribonucleotide reductase (M1 subunit only), dihydrofolate reductase and dihydropyrimidine dehydrogenase cDNAs. 36 unrelated individuals were screened using 6 SSCP conditions and DNA sequencing. Other investigators have identified variances in MTHFR, methionine synthase and folate receptor. These findings are summarized in Table 3.

Table 3: Variation in Genes Which Modulate 5-FU/FA Pharmacology

Gene Name (Genbank accession no.)	Variances			Heterozygote Frequency	Comments
	Base	RNA	Protein		
Cytidine Deaminase (L27943)	79	T or G	lys27glu	>10%	
Dihydrofolate Reductase (J00140)	721	T or A		20%	
	829	C or T		14%	
	RsaI RFLP			23, 33, 43%	3 alleles
	ScrF1			26%	
	RsaI RFLP			32%	unique RsaI RFLP
Dihydropyrimidinase (D78011)	1001	A or G	gln334arg	rare	All found in patients with DHP deficiency
	1303	G or A	gly435arg		
	203	G or C	thr68arg		
	1468	G or C	arg490thr		
	1078	T or C	trp360arg	rare	
	812 to 814	Insertion A	premat. term.		

Dihydropyrimidine Dehydrogenase (U09178)	166 577 3925 3937 3432 638 784 296 to 299 1682 1708 exon/intron 14 1897 2275 2738 3002 2983	T or C A or G A or G T or C T or C A or G C or T Delete TCAT G or A A or G G or A delete C G or A G or A A or T G or T	cys29arg met166val 3' UTR 3' UTR 3' UTR arg21gln val335leu tyr186cys arg235trp premat. term. ser534asn ile543val del. 581-635 premat. term. val732ile arg886his asp974trp val995phe	11% 9% 35% 38% 10% rare rare 2% rare rare 0.5-3% 7-35% 1% rare 1-7% rare rare rare	73% in DPD deficiency
Folate Receptor • • • • • • • •					One Msp I and 2 Pst I RFLPs
Folate receptor • • • • • • • •	330-331	2 bp deletion	Premat. Term.	75%	
Folate Transporter (SLC19A1) (U19720)	341	C or G	Silent	1%	
Folylpolyglutamate Synthetase (M98045)	1747 1900	G or T T or C	3' UTR 3' UTR	2% 50%	
Glycine cleavage System: protein H (M69175)	710	C or G	3' UTR	7%	
Glycine cleavage System: protein P (M64590)			ser564ile	rare	70% in NKH patients
Glycine cleavage System: protein T (D13811)	277 1073 1083 1773	G or T G or A G or A C or T	Val50leu Arg315lys Silent 3'UTR	2% 1% 2% 3%	
Methenyltetrahydro-folate cyclohydrolase	454 969 1614 2011	G or A C or G C or T G or A	Arg134lys Gln306glu Silent Arg653gln Arg293his	22% 1% 1% 35% rare	
Methylenetetra-hydrofolate Reductase (U09806)	129 677 1068 1298 308	C or T C or T C or T C or A T or C	 Ala223val Ala430glu silent	Low 48% low high 5-39% rare	Both the amino acid changes affect MTHFR activity. Rare mutations found in MTHFR deficiency
Methionine Synthase (U50929, U73338))	2756 3970 1158 1004	G or A T or C G or A G or T	Asp919gly Silent Cys225try Ala to ser	19-29% rare rare	Affects folate levels in colon cancer patients. Rare mutations found in MS deficiency
Nucleoside Diphosphate kinase B (X58965)			BgII RFLP		

Ribonucleotide Reductase, M1 (X59543)	1037 2410 2419 2717 2724	C or A A or G A or G T or A T in/del	 SacI RFLP	33% 40% 20% 19% 19% 47%	
Ribonucleotide Reductase, M2 (X59618)	524 1636 2259	C or G C or T T or C	Silent 3' UTR 3' UTR	1% 1% 1%	
Serine Hydroxy-methyltransferase (cytolic) (L11931)	1444 1541	 C or T	Leu474phe 3' UTR	23% 26%	
Thymidine kinase 1 (K02581)	90 279 282 772 867	T or C G or A G or A G or A G or A	Silent Silent Silent 3' UTR 3' UTR Tac1 RFLP BstEII RFLP	50% 13% 30% 26% 50% 40% 2, 34, 64 %	3 alleles
Thymidine kinase 2 (U77088)	1480	T or C	3' UTR	9%	
Thymidine Phosphorylase (PD-ECGF) (S72487)	601 3673 3576	G or C A or G T or C	3' UTR silent	3% 54% rare	 Rare mutations found in MNGIE patients
Thymidylate Synthase (X02308)	276 1140 1210 1571	T or C C or T A or G A or T 28-34 nt repeats	tyr33his 5' reg. Region	rare 53% 42% 53% double: 19%	
Uridine mono-Phosphate synthetase (J03626)	742 1575	G or C A or G	Gly213ala 3' UTR	23% 1% rare	 Rare mutations found in Orotic aciduria patients

A more complete catalog of genetic variances is shown in the following table for the dihydropyrimidine dehydrogenase (DPD) gene.

Table 4

Variances in Dihydropyrimidine Dehydrogenase Gene

Variant nucleotide (codon)	Variant base 1 (frequency)	Variant base 2 (frequency)	Effect on mRNA & protein	Comments
166 (29)	T (62/70)	C (8/70)	cys29arg	Arg allele has no activity when expressed in E. Coli (Vreken, Human Genetics, 1997)
577 (166)	A (69/72)	G (3/72)	met166val	Located in highly conserved domain; no functional studies
784 (235)	C	T	arg235trp	Trp allele has no activity when expressed in E. Coli (Vreken, Human Genetics, 1997)
1682 (534)	G (148/150)	A (2/150)	ser534asn	Apparently little or no functional effect in patient cells.
1708 (543)	A (34/46)	G (12/46)	ile543val	Apparently little or no functional effect in patient cells.
				55 missing amino acids result in

intron 13 (destroys 5' GT splice site immediately after nt 1986)	G	A	no exon 14	unstable protein. Mutant allele may be present in ~1% of Finns; very rare in other groups, but detected in 8 of 11 patients with complete deficiency.
1897 (606)	-	deletion of C	frameshift	Low/no activity allele; reported in only one patient so far.
2738 (886)	G	A	arg886his	His allele has ~25% of normal activity when expressed in Coli (Vreken, Human Genetics, '97)
3002 (974)	A	T	asp974val	Val allele apparently has very low or no activity in patient sample. Very low frequency allele (<0.2% in Americans).
3925	A (41/62)	G (21/62)	3' UTR	Two high frequency variances, 12 nt apart but not in complete linkage disequilibrium.
3937	C (40/64)	T (24/64)	3' UTR	

Variances in the exemplary genes above which affect the activity of the corresponding gene product have the potential to modulate the activity of 5-FU/FA and thereby provide predictive capability concerning the efficacy of such treatment in a particular patient. As discussed above, such predictive capability can further be provided by the joint determination of multiple variances, in one or a plurality of genes or both. Similarly, such variances can provide such predictive capability for other treatments, e.g., treatments with other compounds, which involve these genes.

Example 3 Relationship of Genes to Drug Response – 5-flurouracil

5-fluorouracil (5-FU) is a widely used chemotherapy drug. The effectiveness of 5-FU is potentiated by folinic acid (FA; generic name: leukovorin). The combination of 5-FU and FA is standard therapy for stage III/IV colon cancer. Patient responses to 5-FU and 5-FU/FA vary widely, ranging from complete remission of cancer to severe toxicity.

Clinical Use and Effectiveness of 5-FU and 5-FU/FA

5-FU is a pyrimidine analog in clinical use since 1957. 5-FU is used in the standard treatment of gastrointestinal, breast and head and neck cancers. Clinical trials have also shown responses in cancer of the bladder, ovary, cervix, prostate and pancreas. The remainder of this discussion will concern colorectal cancer. 5-FU is used both in the adjuvant therapy of Dukes Stage B and C cancer and in the treatment of disseminated cancer. 5-FU alone produces partial remissions in 10-30% of advanced colorectal cancers, however only a few percent of patients have complete remissions, and no benefit in survival has been demonstrated.

In the last 15 years a variety of biochemically motivated strategies for

modulating 5-FU activity have been tested. For example, 5-FU has been used in combination with PALA, a pyrimidine synthesis inhibitor, to deplete cellular pools of UTP and thereby enhance formation of FUTP; in combination with methotrexate, to inhibit purine anabolism, leading to increased PRPP levels and consequent increased conversion of 5-FU to its active nucleotide metabolites; and in combination with folinic acid, which increases intracellular pools of reduced folate, driving formation of the ternary inhibitory complex formed by 5,10 methylenetetrahydrofolate, FdUMP and thymidylate synthase. Levamisole, interferon and alkylating agents have also been used in combination with 5-FU. 5-FU/Levamisole and 5-FU/FA are widely used in the adjuvant treatment of colon cancer, while 5-FU/FA is the most commonly used regimen for advanced colorectal cancer. Six of seven prospective randomized trials of 5-FU/FA vs. 5-FU alone in patients with advanced cancer have demonstrated up to two fold higher response rates to 5-FU/FA, while two of the studies also showed increased survival.

Two major dosing regimens are used: 5-FU plus low dose FA given for five consecutive days followed by a 23 day interval, or once weekly bolus iv 5-FU plus high dose FA. The higher FA dose results in plasma FA concentrations of 1 to 10 uM, comparable to those required for optimal 5-FU/FA synergy in tissue culture, however low dose FA (20 mg/m^2 vs. 500 mg/m^2) has produced comparable clinical benefit. Ongoing clinical trials are designed to further test new drug combinations. In summary, relatively few patients - in the single digits - live longer as a result of 5-FU/FA, although significantly more have partial disease remission. The factors that determine which patients respond or have side effects are not known.

5-FU modulators

Leukovorin (folinic acid) is the most widely used 5-FU modulator, however a variety of other molecules have been used with 5-FU, including, for example, interferon-alpha, hydroxyurea, N-phosphonacetyl-L-aspartate, dipyridamole, levamisole, methotrexate, trimetrexate glucuronate, cisplatin and radiotherapy. S-1 is a novel oral anticancer drug, composed of the 5-FU prodrug tegafur plus gimestat (CDHP) and otastat potassium (Oxo) in a molar ratio of 1:0.4:1, with CDHP inhibiting dihydropyrimidine dehydrogenase in order to prolong 5-FU concentrations in blood and tumour and Oxo present as a gastrointestinal protectant. Some of these regimens show promising results, but no clear improvement over 5-FU/leukovorin. The clinical development and use of regimens containing 5-FU plus modulators may be facilitated by the methods of this invention.

Toxicity of 5-FU and Folinic Acid

5-FU toxicity has been well documented in randomized clinical trials. Patients receiving 5-FU/FA are at even greater risk of toxic reactions and must be monitored carefully during therapy. A variety of side effects have been observed, affecting the gastrointestinal tract, bone marrow, heart and CNS. The most common toxic reactions are nausea and anorexia, which can be followed by life threatening mucositis, enteritis and diarrhea. Leukopenia is also a problem in some patients, particularly with the weekly dosage regimen. In a recent randomized trial of weekly vs. monthly 5-FU/FA, there were 7 deaths related to drug toxicity among 372 treated patients (1.9%; Buroker et al. 1994). 31% of patients receiving the weekly regimen suffered diarrhea requiring hospitalization for a median of 10 days. Other severe toxicities, which occured at lower frequency, included leukopenia and stomatitis. In another example, 36% of patients receiving weekly bolus 5-FU plus FA (500 mg/m^2), in a NSABP trial suffered NCI grade 3 toxicity (Wolmark et al., 1996). Clearly, toxicity is a major cost of 5-FU/FA therapy, measured both in patient suffering and in financial terms (the cost of care for drug induced illness).

Other Factors

Many non-genetic factors can influence the response of cancers to drugs, including tumor location, vasculature, cell growth fraction and various drug resistance mechanisms. It is therefore not possible to explain all heterogeneity in response to 5-FU/FA administration by genetic variation. However, based on genetic studies of other quantitative traits it appears that a significant fraction of variation in drug response is due to genetic variation.

Example 4 Genetic Component of Drug Response Variability
Genetically Determined Variation in Response to 5-FU: Studies of Dihydropyrimidine Dehydrogenase Deficiency

Dihydropyrimidine Dehydrogenase Deficiency is Associated with 5-FU Toxicity

5-FU is inactivated by the same metabolic pathway as thymine and uracil (see above). DPD catalyzes the first, rate limiting step in pyrimidine catabolism and accounts for elimination of most 5-FU. Normal individuals eliminate 5-FU with a half life of ~10-15 minutes and excrete only 10% of a dose unchanged in the urine. In contrast, people genetically deficient in DPD eliminate 5-FU with a half life of ~2.5 hours and excrete 90% of a dose unchanged in the urine (Diasio et al., 1988). DPD deficiency has two clinical presentations: (i) an inborn error of metabolism causing

some degree of neurologic dysfunction or (ii) asymtomatic until revealed by exposure to 5-FU or other pyrimidine analogs. With either presentation there is combined hyperuraciluria and hyperthyminuria. The vastly increased 5-FU half life in DPD deficient individuals causes severe toxicity and even death. Recently several mutations have been identified in DPD genes of deficient individuals (Wei et al., 1996), however none of these alleles appears to occur at appreciable frequency, so the cause of wide population variation in DPD levels is still not understood.

Dihydropyrimidine dehydrogenase (DPD) inhibitors

More than 85% of an injected dose of 5-FU is rapidly inactivated by dihydropyrimidine dehydrogenase (DPD) to therapeutically inactive catabolic products, however there is evidence that said catabolic products may be toxic to normal tissues. This has led to the development of DPD inhibitors with the aim to modify the therapeutic index of 5-FU. Several inhibitors in combination with 5-FU are under preclinical and clinical evaluation, including uracil and 5-chloro-2,4-dihydroxy pyridine, as modulators of 5-FU derived from its prodrug tegafur and 5-ethynyluracil as a modulator of 5-FU itself (Eniluracil, 776C85; Glaxo Wellcome Inc, Research Triangle Park, NC). Other compounds with DPD inhibitory activity include 5-propynyluracil. (For a review of DPD inhibitors see: Diasio, RB Improving 5-FU with a Novel Dihydropyrimidine Dehydrogenase Inactivator, Oncology 1998, Mar; 12(3 Suppl. 4):51-6.)

Population Studies of DPD Activity Show Wide Variation

Population surveys of DPD activity in normal individuals have been performed using blood and liver samples. These studies reveal a broad unimodal Gaussian distribution of DPD activity over a 7 to 14 fold range, with some individuals having very low or even undetectable levels. For example Etienne et al. (1994) report DPD activity ranging from .065 to .559 nM/min/mg protein in a study of 152 men and 33 women, while Fleming et al. (1993) found DPD activity in 66 cancer patients varied from .17 to .77 nM/min/mg protein. Lu et al (1995) found 18-fold variation in liver DPD assayed in 138 individuals. Milano and Etienne (1994) suggested that the frequency of heterozygous and homozygous deficiendy is 3% and .1%, respectively. The DNA sequence alterations responsible for null DPD alleles do not account for the high population variability (Ridge et al., 1997).

DPD Levels Correlate with Response to 5-FU

Intratumoral DPD levels have been measured in patients receiving 5-FU chemotherapy. When complete responders were compared to partial or nonresponders,

DPD levels were lower in the compete responders (Etienne et al., 1995). Leukocyte DPD levels have also been measured in patients receiving 5-FU/FA chemotherapy. When patients were divided into 3 groups: high, medium and low DPD activity, the frequency of serious side effects was highest in the low DPD group and vice versa (Katona et al., 1997).

Biochemical Studies of Alternate Allelic Forms of DPD

The power of genetic analysis can be augmented by biochemical studies of alternate allelic forms of enzymes. Biochemical data on the distribution of activity of a series of enzymes in a biochemical pathway provides the basis for metabolic flux analysis (Keightly, 1996). It is beyond the scope of this proposal to exhaustively analyze biochemical variation in the enzymes of pyrimidine and folate metabolism. However, since we have identified new variances in DPD that may affect enzyme expression or activity, and because DPD is already proven to play a role in 5-FU response, we will determine the relationship between genotype and biochemistry for this enzyme.

DPD cDNAs have been cloned from a variety of higher eukaryotes and binding sites for its cofactors, prosthetic groups and substrate have been defined experimentally or by analogy with known consensus motifs (Yokata et al., 1994). The DPD polymorphisms that affect protein sequence occur at amino acids 29 (cys/arg) and 166 (met/val) in the amino-terminal one-third of the protein. Phylogenetic comparison of this region from boar, human, cow, fly, and bacteria (see below) shows that there are actually two highly conserved motifs that resemble either iron/sulfur or zinc binding motifs, the latter being more likely due to the spacing of the cysteine residues. The region around the met/val polymorphism at amino acid 166 is highly conserved. Even the spacing of the putative zinc-finger domains is maintained between distantly related species, hinting at their importance. Since amino acid 166 is close to a highly conserved (and probably functionally important) region and is itself conserved, being a methionine in all species, it seems likely that perturbations in this position would have consequence. The polymorphism substitutes a long amino acid side chain capable of hydrogen bonding (methionine) for a compact, hydrophobic amino acid (valine). The region around amino acid 29 is not as well conserved.

Common DPD Haplotypes

Eight haplotypes from 58 chromosomes (29 individuals) have been identified. Using methods described above, the DNA from these samples were analyzed by PCR. The single base pair substitutions at four locations were identified as allelic haplotypes,

e.g. base pair number 166, 577, 3925, 3937. Base pair positions, 3925 and 3937 are located in the 3 prime untranslated region of the cDNA and base pairs 166 and 577 are within the coding region.

Table 5

Identified DPD Haplotypes

	Base Position			
No. Chromosomes	166	577	3925	3937
14 (24%)	T (cys)	A (met)	G	C
16 (28%)	T (cys)	A (met)	A	C
16 (28%)	T (cys)	A (met)	A	T
4 (7%)	C (arg)	A (met)	A	T
3 (5%)	C (arg)	A (met)	G	C
3 (5%)	C (arg)	A (met)	A	C
1 (2%)	T (cys)	G (val)	G	C
1 (2%)	T (cys)	G (val)	A	C
Total=58(100%)				

Example 5 – Exemplary Genes involved in Folate Transport and Metabolism

While examples above concern 5-FU/FA action and genes which are expected to modulate such action, it is also useful to utilize genes involved in folate transport and metabolism generally. A number of these genes are also involved in 5-FU/FA action. Genes known to be involved in folate transport and metabolism are listed in the table below, along with available GenBank accession numbers for deposited sequences.

Table 6
Gene Field: Folate Transport & Metabolism

Folate Transporters	**Folate Polyglutamation**	**Biosynthesis, Degradation and Interconversion of Folates**	
Folate receptor 1(•) (GenBank M28099)	Folylpolyglutamate synthetase (GenBank M98045)	Formiminotetrahydrofolate cyclodeaminase	Glutamate formiminotransferase
Folate receptor (•) (GenBank J02876)		Methenyltetrahydrofolate synthetase	Formyltetrahydrofolate hydrolase
Folate receptor (•) (GenBank Z32564)		Methylenetetrahydrofolate dehydrogenase	Methylenetetrahydrofolate synthase GenBank L38298

Folate Transporter (SLC19A1) GenBank U19720		*Methionine synthetase* GenBank U50929	*Methylenetetrahydrofolate reductase* GenBank U09806
Folate Absorbtion	**Inhibition of dTMP Synthesis**	*Dihydrofolate reductase* GenBank J00140	Serine transhydroxy-methylase 1 GenBank L11931
Pteroyl-• -glutamyl carboxypeptidase	*Thymidylate synthase* GenBank X02308	Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase (one locus) GenBank J04031	Glycine cleavage system, Protein H: GenBank M69175; Protein P: GenBank M64590; Protein T: GenBank D13811; Protein L Formyltetrahydrofolate dehydrogenase

Genes affecting the action of drugs which modulate folate metabolism.

There are 24 genes in the Table, four of which we have already surveyed for polymorphism (*italicized genes*). The genes with GenBank numbers are currently being screened for variances. Genes lacking GenBank numbers are not yet represented in GenBank as full length cDNAs; but will be scanned using relevant EST collections or using sequences from other publicly available sources.

Example 6 – Drugs Targeting Genes Involved in Folate Transport and Metabolism

In concert with the identification of useful genes involved in folate transport and metabolism, the table below identifies certain drug classes used for treatment of identified disorders, along with a brief characterization of the action of the drug. Exemplary drugs are identified within the individual classes. Variable response of patients to administration of drugs of these classes, or administration of the specific drugs can be used in identifying variances responsible for such variable response. As described above, those variances can then be used in diagnostic tests, methods of selecting a treatment, methods of treating a patient, or other methods utilizing genetic variance information as otherwise described.

Table 7

Drug Field: Folate Transport & Metabolism

Disease/ Indication	Drug Class	Mechanism of Action	Exemplary Drugs
Cancer	Reduced folates	Block dTMP biosynthesis by inhib-iting thymidylate synthase (TS) via formation of ternary complex invol-ving TS, 5-fluorodeoxyuridine and 5,10-methylenetetrahydrofolate	leukovorin, L-leu-kovorin, citrovor-um factor (used with 5-fluorouracil or related drugs)
Cancer	Reduced folates	Rescue bone marrow from lethal toxicity after high dose methotrexate	leukovorin, L-leu-kovorin, citrovor-um factor

Cancer	Folate analogs (antifolates)	Block *de novo* purine biosynthesis by inhibiting dihydrofolate reduc-tase, TS,	Methotrexate, aminopterin, dide-azatetrahydrofolate
Proliferative skin diseases (psoriasis)	Folate analogs (antifolates)	Block *de novo* purine biosynthesis by inhibiting dihydrofolate reduc-tase, TS,	Methotrexate, aminopterin, dide-azatetrahydrofolate
Immunosup-pression	Folate analogs (antifolates)	Block *de novo* purine biosynthesis by inhibiting dihydrofolate reduc-tase, TS,	Methotrexate, aminopterin, dide-azatetrahydrofolate
Autoimmune diseases, such as rheumatoid arthritis	Folate analogs (antifolates)	Block *de novo* purine biosynthesis by inhibiting dihydrofolate reduc-tase, TS,	Methotrexate, aminopterin, dide-azatetrahydrofolate
Folate deficiency	Folic acid	Increase folates for purine and pyrimidine biosynthesis	Folic acid
Cardiovascular disease (prevent atherosclerosis)	Folic acid	Reduce plasma homocysteine levels in patients with low MTHFR levels	Folic acid
Prevent spina bifida	Folic acid	Reduce plasma homocysteine levels in patients with low MTHFR levels	Folic acid

Table 7. Drugs which affect or are affected by folate metabolism. A wide spectrum of diseases are treated with drugs that affect folate metabolism. Some drugs are used in the treatment of several diseases. All of the listed drugs are frequently used in combination with other drugs. For example methotrexate is used in cancer chemotherapy with cytoxan and fluoruracil to treat breast cancer, among other combinations.

Folate analogs

Many novel antifolate compounds with unique pharmacologic properties are currently in clinical development. These newer antifolates differ from methotrexate, the most widely used and studied drug in this class, in terms of their lipophilicity, cellular transport mechanism, level of polyglutamation, and specificity for inhibiting folate-dependent enzymes, such as dihydrofolate reductase, thymidylate synthase, or glycinamide ribonucleotide formyltransferase. The clinical development and use of these new compounds can be affected by the methods of this invention. The new folate analogs include quinazoline derivatives such as ZD1694 (Tomudex, AstraZeneca) which requires Reduced Folate Carrier (RFC) mediated cell uptake and polyglutamation by Folylpolyglutamate Synthetase (FPGS); ZD9331 (AstraZeneca), which requires the RFC but is not polyglutamated by FPGS; LY231514 (Eli Lilly Research Labs, Indianapolis, IN) is a multitargeted pyrrolopyrimidine analogue antifolate which requires the RFC and polyglutamation; GW1843 (1843U89, GlaxoWellcome) is a benzoquinazoline compound with potent TS inhibitory activity, and which enters cells via the RFC but is polyglutamated only to the diglutamate, which leads to higher cellular retention without augmenting TS inhibitory activity; AG337 (p.o. and i.v. forms) and AG331 (both by Agouron, La Jolla, CA, now part of Warner Lambert) are

lipophilic TS inhibitors with action independent of the RFC and polyglutamation by FPGS; trimetrexate (US Bioscience) is a ; Aminopterin is an older drug which has received renewed attention recently; edatrexate, piritrexim and lometrexol are other antifolate drugs. More generally, 5,8-dideazaisofolic acid (IAHQ), 5,10-dideazatetrahydrofolic acid (DDATHF), and 5-deazafolic acid are structures into which a variety of modifications have been introduced in the pteridine/quinazoline ring, the C9-N10 bridge, the benzoyl ring, and the glutamate side chain (see article below). Also Lilly have recently synthesized a new series of 2,4-diaminopyrido[2,3-d]pyrimidine based antifolates which are being evaluated both as antineoplastic and antiarthritic agents.

Other Therapeutic Categories in which Folate or Pyrimidine Pathwyas may be Relevant to Drug Development

1) Cardiovascular Drugs

Homocysteine is a proven risk factor for cardiovascular disease. One important role of the folate cofactor 5-methyltetrahydrofolate is the provision of a methyl group for the remethylation of homocysteine to methionine by the enzyme methionine synthase. Variation in the enzymes of folate metabolism, for example methionine syntase or methylenetetrahydrofolate reductase (MTHFR), may affect the levels of 5-methyltetrahydrofolate or other folates that in turn influence homocysteine levels. The contribution of elevated homocysteine to atherosclerosis, thromboembolic disease and other forms of vascular and heart disease may vary from one patient to another. Such variation may be attributable, at least in part, to genetically determined variation in the levels or function of the enzymes of folate metabolism described in this application. Assistance of clinical development or use of drugs to treat said cardiovascular diseases might be afforded by an understanding of which patients are most likely to benefit. This is true whether the drugs are aimed at the modulation of folate levels (e.g. supplemental folate) or at other known causes of cardiovascular disease (e.g. lipid lowering drugs such as statins, or antithrombotic drugs such as salicylates, heparin or GPIIIa/IIb inhibitors). It may, for example, be desirable to exclude patients whose disease is significantly attributable to elevated homocysteine from treatment with agents aimed at the amelioration of other etiological causes, such as elevated cholesterol. Thus, the understanding of variation in the enzymes of folate transport and metabolism may be important in evaluating drugs used to treat atherosclerosis, thromboembolic diseases and other forms of vascular and heart disease.

2) CNS drugs

The observation that phencyclidine, an NMDA receptor antagonist, induces a psychotic state closely resembling schizophrenia in normal individuals has led to attempts to modulate NMDA receptor function in schizophrenic patients. The amino acid glycine is an obligatory coagonist (with glutamate) at NMDA receptors (via its action at a strychnine-insensitive binding site on the NMDA receptor complex), and consequently glycine or glycinergic agents (e.g. glycine, the glycine receptor partial agonist, D-cycloserine, or the glycine prodrug milacemide) have been tried as an adjunct to conventional antipsychotics for the treatment of schizophrenia. Several trials have demonstrated a moderate improvement in negative symptoms of schizophrenia. Because the folate pathway modulates levels of serine and glycine, the endogenous levels of glycine in neurons may affect the response to glycine or glycinergic drugs. In particular, interpatient variation in glycine metabolism may affect drug efficacy.

Example 7 – Genes Related to Pyrimidine Transport and Metabolism

Similar to the genes involved in folate transport and metabolism, genes involved in the related pathways of pyrimidine transport and metabolism are useful in the aspects of the present invention, e.g., for identifying variances responsible for variable treatment response, diagnostic methods, and methods of selecting a patient to receive a treatment. Exemplary genes are provided below and are further identified by cellular function. Genes involved in those functions are generally useful in the present invention.

Table 8
Gene Field: Pyrimidine Transport & Metabolism

Pyrimidine Transport	Pyrimidine Biosynthesis - *de novo* and Salvage Pathways		Pyrimidine Catabolism
Equilibrative nucleoside transporter 1	Uridine phosphorylase GenBank X90858	Ribonucleoside reductase: *M1 subunit* GenBank X59543 M2 subunit GenBank X59618	*Dihydropyrimidine Dehydrogenase* GenBank U09178
Equilibrative nucleoside transporters 2, 3, 4 & 5	Thymidine phosphorylase GenBank S72487	Nucleoside diphosphate kinase, A subunit GenBank U29200	Dihydropyrimidinase GenBank D78011
Concentrative nucleoside transporters	Orotate phosphoribosyl-transferase GenBank J03626	B subunit GenBank X58965	• -ureidopropionase
	Uridine Kinase GenBank D78335	Uridine mono-phosphate kinase	Cytidine deaminase

	Thymidine kinase GenBank K02581; Thymidine Kinase 2 GenBank U77088	Deoxycytidylate kinase	dCMP deaminase
Inhibition of dTMP Synthesis	Deoxycytidine kinase		β-alanine-pyruvate aminotransferase
Thymidylate synthase GenBank X02308			β-alanine-α-detoglutarate aminotransferase

Table 8. Genes affecting the action of drugs which modulate pyrimidine metabolism. We have already surveyed three of the above genes for polymorphism (*italicized genes*). The genes with GenBank numbers are currently being screened for variances. Genes in the table lacking GenBank numbers are not yet represented in GenBank as full length cDNAs; but can be evaluated using relevant EST collections. Genes not listed in the Table but related to the mechanism of action of pyrimidine analogs include DNA and RNA polymerases and subunits and DNA repair enzymes, some of which (e.g. DNA polymerase • and 220 kD and 33 kD subunits of RNA polymerase II) have already been screened for polymorphism. Such additional genes can also be used in the present invention.

Example 8 – Drugs Targeting Genes Involved in Pyrimidine Transport & Metabolism

As was described above for drugs modulating genes involved in folate transport and metabolism, particular drug classes and exemplary drugs are identified in the table below which modulate the action of pyrimidine transport and metabolism genes. These classes of drugs and exemplary drugs are similarly useful for identifying variances which affect the action

Table 9
Drug Field: Pyrimidine Transport & Metabolism

Disease/ Indication	Drug Class	Mechanism of Action	Exemplary Drugs
Cancer	Fluoropyrimidines	Block dTTP biosynthesis by inhib-iting thymidylate synthase; inhibit replication, transcription and/or repair by incorporation into DNA and RNA.	5-FU, fluorode-oxyuridine, flu-orodeoxyuridine monophosphate, tegafur, ftorafur.
Cancer	Dihydropyrimidine dehydrogenase inhibitors	Potentiate fluoropyrimidines by blocking their catabolism, increasing half life.	5-ethynyluracil; 5-propynyluracil; 2,6 dihydroxypy-ridine
Cancer	Cytidine analogs	Incorporation into DNA and conse-quent inhibition of DNA synthesis (replication, transcription, repair).	Cytosine arabino-side, gemcitabine, 5-azacytidine, 5-azacytosine ara-binoside, others.

Cancer	Other pyrimidine analogs	Inhibition of nucleic acid synthesis	
Cancer	Ribonucleotide reductase inhibitors	Inhibit reduction of ribonucleotides (e.g. CTP) to deoxyribonuc-leotides (dCTP)	Hydroxyurea
Cancer	Nucleotide/nucleoside uptake inhibitors	Block import of cytotoxic pyrimi-dine analogs (protective effect), or block import of normal pyrimidine nucleotides, thereby reducing salvage synthesis and increasing need for de novo synthesis, including dTMP synthesis.	dipyridamole, BIBW 22 (a dipyridamole analog), nitroben-zylthioinosine

Table 9. Genes affecting the action of drugs which modulate pyrimidine metabolism. A variety of proliferative diseases, especially cancer, are treated with drugs that affect pyrimidine metabolism. All of the listed drugs are frequently used in combination with other drugs.

Other Pyrimidine Analogs

There are a large number of pyrimidine analogs in clinical development for a wide variety of indications. One of the most common indications is cancer and leukemia and lymphoma of various types. For example, 2',2'-difluorodeoxycytidine (gemcitabine; Gemzar) is a pyrimidine nucleoside drug with clinical efficacy in several common solid cancers; cytosine arabinoside (ARA-C) is another pyrimidine analog used in the treatment of leukemia; 2-chlorodeoxyadenosine and fludarabine (F-araA) are also used as antineoplastic drugs. 2'-deoxy-2'-(fluoromethylene) cytidine (MDL 101,731, Kyowa Hakko Kogyo Co.), 2',2'-difluorodeoxycytidine, 5-aza-2'deoxycytidine (decitabine), 5-azacytidine, 5-azadeoxycytidine, and __ are under development as antineoplastic drugs.

CNS Drugs – Pyrimidine Pathway

The pyrimidine nucleoside, uridine, has been proposed as a potential supplement in the treatment of psychosis based on its ability to reduce haloperidol-induced dopamine release. Thus, coadministration of uridine with haloperidol might enhance the antipsychotic action of standard neuroleptics, allowing for a reduction in dose and thereby a reduction in the frequency of side effects. The presumed mechanism is interaction with dopamine or GABA neurotransmission. The levels or function of pyrimidine transporters or pyrimidine *de novo* or salvage biosynthetic enzymes, or pyrimidine catabolic enzymes may affect the action of neuroleptics, or their modulation by pyrimidine nucleosides or pyrimidine analogs.

Other Therapeutics Relevant to the Pyrimidine Pathway

Another possible mode of pyrimidine nucleotide action is via stimulation of thromboxane A2 release from cultured glial cells. Uridine triphosphate, uridine

diphosphate, cytidine triphosphate, and deoxythymidine triphosphate all induce concentration-dependent increases in the release of thromboxane A2 from cultured glial cells, indicating a possible role in brain response to damage *in vivo*.

Other cancers such as head and neck, breast, pancreas, other gastrointestinal cancers including stomach and intestinal may be directly targeted by therapeutic intervention that affects DNA methylation levels, pyrimidine synthesis, transport, and degradation pathways.

Many neurological diseases in both the CNS and the periphery may also be affected by therapeutic intervention of DNA methylation, pyrimidine synthesis, transport, and degradation pathways. Such intervention may be of therapeutic benefit to halt, retard, and or reduce symptoms of these often debilitating diseases.

Example 9 – Drugs That Affect the Folate and Pyrimidine Pathways

There are many potential candidate therapeutic interventions or drugs that can affect the folate and pyrimidine pathways. Categories of these are 5-FU prodrugs, drugs that affect DNA methylation pathways, and other drugs that have been developed for similar indications as 5-FU.

5-FU prodrugs

The clinical development and use of 5-FU prodrugs is further subject to improvement by the methods of this invention. These drugs are generally modified fluoropyrimidines that require one or more enzymatic activation steps for conversion into 5-FU. The activation steps may result in prolonged drug half-life and/or selective drug activation (i.e. conversion to 5-FU) in tumor cells.

Examples of such drugs include capecitabine (Xeloda, Roche), a drug that is converted to 5-FU by a three-step pathway involving Carboxylesterase 1, Cytidine Deaminase and Thymidine Phosphorylase. Another 5-FU prodrug is 5'deoxy 5-FU (Furtulon, Roche) which is converted to 5-FU by Thymidine Phosphorylase and/or Uridine Phosphorylase. Another 5-FU prodrug is 1-(tetrahydro-2-furanyl)-5-fluorouracil (FT, ftorafur, Tegafur, Taiho - Bristol Myers Squibb), a prodrug that is converted to 5-FU by cytochrome P450 enzyme, CYP3A4.

Drugs acting on DNA methyation pathways

Antivirals

Herpes virus thymidine kinase phosphorylates many 5-substituted 2'-deoxyuridines, analogs of thymidine (e.g., idoxuridine, trifluridine, edoxudine, brivudine) and 5-substituted arabinofuranosyluracil derivatives (e.g., 5-Et-Ara-U, BV-

Ara-U, Cl-Ara-U). The 5'-monophosphates are further phosphorylated by cellular enzymes to the 5'-triphosphates, which are usually competitive inhibitors of the viral-coded DNA polymerases.

Unlike herpes viruses, retroviruses including but not limited to human immunodeficiency viruses do not encode specific enzymes required for the metabolism of the purine or pyrimidine nucleotides to their corresponding 5'-triphosphates. Therefore, 2',3'-dideoxynucleosides and acyclic nucleoside phosphonates must be phosphorylated and metabolized by host cell kinases and other enzymes of purine and/or pyrimidine metabolism. In this way, affecting the pyrimidine synthetic, transport, or degradation pathways by candidate therapeutic intervention may be therapeutic beneficial in treating retroviral infections. Excamples of candidate antivirals that may be affected by alteration of pyrimidine synthetic, transport, or degradation pathwyas are azidothymidine (AZT), acyclovir, and ganciclovir. These and other drugs have been used both as antivirals and antineoplastic agents.

Other Drugs Developed for Similar Indications as 5-FU

A variety of drugs are being developed for similar indications as 5-FU, and/or are being tested in combinations with 5-FU/leukovorin. These include the new platinum compound oxaliplatin (L-OHP) and the topoisomerase I inhibitors irinotecan (CPT11, Pharmacia-UpJohn) and topotecan. The effective clinical development or clinical use of these drugs may be enhanced by the methods of this invention. In particular, identification of patients likely to respond to 5-FU with or withour leukovorin, may be useful in selecting optimal responders to other drugs. Alternatively identification of patients likely to suffer toxic response to 5-FU containing regimens may allow identification of patients best treated with other drugs. Other drugs with activity against cancers usually treated with regimens containing 5-FU (e.g. metastatic colon cancer) include Suramin, a bis-hexasulfonated napthylurea; 6-hydroxymethylacylfulvene (HMAF; MGI 114); LY295501; bizelesin (U-7779; NSC615291), ONYX-015, monoclonal antibodies (e.g. 17-1A and MN-14), protein synthesis inhibitors such as RA 700, and angiogenesis inhibitors such as PF 4. Still other drugs may prevent colorectal cancer by preventing the formation of colorectal polyps (eg, cyclooxygenase inhibitors may induce apoptosis of polyps).

Example 10

Protocol for Clinical Trial for Determining the Relationship Between Toxicity of a Drug and Genetic Variances in Genes Related to the Action of the Drug

THIS EXAMPLE PROVIDES AN EXEMPLARY CLINICAL TRIAL AS A CASE CONTROL STUDY WHICH INCLUDES EVALUATING THE EFFECTS OF SEQUENCE VARIANCES IN ENZYMES WHICH CAN MEDIATE THE EFFECTS OF A KNOWN DRUG, IN THIS CASE IN AN ANTICANCER TREATMENT. THE INFORMATION IN THE BACKGROUND SECTION OF THIS PROTOCOL IS ALSO PROVIDED IN LARGE PART IN THE DETATILED DESCRIPTION, BUT IS REPEATED HERE FOR COMPLETENESS OF THE PROTOCOL DESCRIPTION.

PROTOCOL TITLE: Case-control study to determine the relationship between toxicity of 5-fluorouracil (5-FU) given with folinic acid (FA) to patients with solid tumors and DNA sequence variances in enzymes that mediate the action of 5-FU and FA.

II. SIGNATURE PAGE

5

10 _____

Name, position, and address of individual approving protocol from study sponsor.

15

_____

Name, position, and address of individual approving protocol from study sponsor.

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III. TABLE OF CONTENTS

IV. ACRONYMS AND ABBREVIATIONS

5-FU	5-Fluorouracil
FA	Folinic acid
°C	Degree centigrade
CBC	Complete blood count
CRF	Case report form
DCC	Data Coordinating Center
DMC	Data Monitoring Committee
EC	Ethical Committee
ECG	Electrocardiogram
e.g.	For example
°F	Degrees Fahrenheit
FDA	Food and Drug Administration
i.e.	That is
IRB	Institutional Review Board
IV	Intravenous
mcg	Microgram
mg	Milligram
mL	Milliliter
mm^3	Cubic millimeter
PD	Pharmacodynamic
PK	Pharmacokinetic
®	Registered trade mark
REB	Research Ethics Board
USA	United States of America
USP	United States Pharmacopoeia

V. *STUDY FLOW CHART*

	File Research	Medical Visit
Selection of patients from the file	X	
Informed Consent Form signed		X
Inclusion/Exclusion criteria checking		X
Chart reporting		X
Demographic reporting		X
Blood sampling		X

5

VI. 1. SUMMARY

Protocol

Title: Case-control study to determine the relationship between toxicity of 5-fluorouracil (5-FU) given with folinic acid (FA) to patients with solid tumors and DNA sequence variances in enzymes that mediate the action of 5-FU and FA.

VII. Study

VIII. Phase: Phase IV

Study

Design: Single-center, case-control study.

Study

Objectives: The primary objective of this study is to compare the variance frequency distribution in the dihydropyrimidine dehydrogenase (DPD) gene between two groups of patients with solid tumors, treated by weekly or monthly regimen of 5-FU+FA and defined by level of toxicity (graded according to the NCI common toxicity criteria) as:

- Group 1: patients with high toxicity (grade III / IV on NCI criteria)
- Group 2: patients with minimal toxicity (grade 0 / I / II on NCI criteria)

The secondary objectives of the study are to determine the DPD gene haplotype frequency distribution and the variance and/or haplotype frequency distributions in selected genes (other than DPD gene) between two groups of patients with solid tumors, treated by weekly or monthly regimen of 5-FU+FA and defined by level of toxicity. Analyses will be done globally, then by regimen (monthly vs. weekly) and by type of toxicity (gastrointestinal vs. bone marrow).

Number of Subjects: Ninety (90) patients, 45 in each group, will be included.

Study Population: Patients treated with 5-FU+FA for solid tumors at the Massachusetts General Hospital, Dana-Farber Cancer Institute and Brigham and Women's Hospital.

StudyGroups: Patients will be divided into two groups depending on the degree of toxicity they experienced with treatment, if any:

- patients with high toxicity (grade III / IV on NCI criteria),
- patients with minimal toxicity (grade 0 / I / II on NCI criteria)

Visit Schedule: One visit to sign the informed consent form and to collect blood sample.

Evaluation Parameter: Frequency distribution of gene alleles and haplotypes.

IX. 2. INTRODUCTION

X. 2.1 Background

XI. 2.1.1 Potential for Improved Effectiveness of 5-FU and 5-FU/FA

Introduction

Chemotherapy of cancer involves use of highly toxic drugs with narrow therapeutic indices. Although progress has been made in the chemotherapeutic treatment of selected malignancies, most adult solid cancers remain highly refractory to treatment. Nonetheless, chemotherapy is the standard of care for most disseminated solid cancers. Chemotherapy often results in a significant fraction of treated patients suffering unpleasant or life-threatening side effects while receiving little or no clinical benefit; other patients may suffer few side effects and/or have complete remission or even cure. Any test that could predict response to chemotherapy, even partially, would allow more selective use of toxic drugs, and could thereby significantly improve efficacy of oncologic drug use, with the potential to both reduce side effects and increase the fraction of responders. Chemotherapy is also expensive, not just because the drugs are often costly, but also because administering highly toxic drugs requires close monitoring by carefully trained personnel, and because hospitalization is often required for treatment of (or monitoring for) toxic drug reactions. Information that would allow patients to be divided into likely responder vs. non-responder (or likely side effect) groups, only the former to receive treatment, would therefore also have a significant impact on the economics of cancer drug use.

Predicting Response to Chemotherapy

Several methods for predicting response to chemotherapy in individual patients have been investigated over the years, ranging from the use of biochemical markers to testing drugs on a patients cultured tumor cells. None of these methods has proven sufficiently informative and practical to gain wide acceptance. However, there are some specific examples of tests useful for predicting toxicity. For example, a diagnostic test to predict side effects associated with the antineoplastic drugs 6-mercaptopurine, 6-thioguanine and azathioprine has begun to gain wide acceptance, particularly among pediatric oncologists. Severe toxicity of thiopurine drugs is associated with deficiency

of the enzyme thiopurine methyltransferase (TPMT). Currently most TPMT testing is done using an enzyme assay, however the TPMT gene has been cloned and mutations associated with low TPMT levels have been identified; genetic testing is beginning to supplant enzyme assays because genetic tests are more easily standardized and economical.

While there are no good tests that predict positive chemotherapeutic response, there is demonstrated utility to measuring estrogen and progesterone receptor levels in cancer tissue before selecting therapy directed at modulating hormonal state. Measuring genetic variation in proteins that mediate the effects of chemotherapy drugs is in some respects analogous to measuring ER and PR levels, which mediate the effects of hormones.

Clinical Use and Effectiveness of 5-FU and 5-FU/FA

5-FU is a pyrimidine analog in clinical use since 1957. 5-FU is used in the standard treatment of gastrointestinal, breast and head and neck cancers. Clinical trials have also shown responses in cancer of the bladder, ovary, cervix, prostate and pancreas. The remainder of this discussion will concern colorectal cancer. 5-FU is used both in the adjuvant therapy of Dukes Stage B and C cancer and in the treatment of disseminated cancer. 5-FU alone produces partial remissions in 10 - 30% of advanced colorectal cancers, however only a few percent of patients have complete remissions. In the last 15 years a variety of biochemically motivated strategies for modulating 5-FU activity have been tested. For example, 5-FU has been used in combination with PALA, a pyrimidine synthesis inhibitor, to deplete cellular pools of UTP and thereby enhance formation of FUTP; in combination with methotrexate, to inhibit purine anabolism, leading to increased PRPP levels and consequent increased conversion of 5-FU to its active nucleotide metabolites; and in combination with folinic acid, which increases intracellular pools of reduced folate, driving formation of the ternary inhibitory complex formed by 5,10 methylenetetrahydrofolate, FdUMP and thymidylate synthase. Levamisole, interferon and alkylating agents have also been used in combination with 5-FU. 5-FU/Levamisole and 5-FU/FA are widely used in the adjuvant treatment of colon cancer, while 5-FU/FA is the most commonly used regimen for advanced colorectal cancer. Several prospective randomized trials of 5-FU/FA vs. 5-FU alone in patients with advanced cancer have demonstrated up to two fold higher response rates to 5-FU/FA, while three of the studies also showed increased survival. Two major dosing regimens are used: 5-FU plus low dose FA given for five consecutive days

followed by a 23 day interval, or once weekly bolus IV 5-FU plus high dose FA. The higher FA dose results in plasma FA concentrations of 1 to 10 uM, comparable to those required for optimal 5-FU/FA synergy in tissue culture, however low dose FA (20 mg/m^2 vs. 500 mg/m^2) has produced comparable clinical benefit. Ongoing clinical trials are designed to further test new drug combinations. In summary, relatively few patients - in the single digits - live longer as a result of 5-FU/FA, although significantly more have partial disease remission. The factors that determine which patients respond or have side effects are not known.

Toxicity of 5-FU and Folinic Acid

5-FU toxicity has been well documented in randomized clinical trials. Patients receiving 5-FU/FA are at even greater risk of toxic reactions and must be monitored carefully during therapy. A variety of side effects have been observed, affecting the gastrointestinal tract, bone marrow, heart and CNS. The most common toxic reactions are nausea and anorexia, which can be followed by life threatening mucositis, enteritis and diarrhea. Leukopenia is also a problem in some patients, particularly with the weekly dosage regimen. In a recent randomized trial of weekly vs. monthly 5-FU/FA there were 7 deaths related to drug toxicity among 372 treated patients (1.9%; Buroker et al. 1994). 31% of patients receiving the weekly regimen suffered diarrhea-requiring hospitalization for a median of 10 days. Other severe toxicity, which occurred at lower frequency, included leukopenia and stomatitis. In another example, 36% of patients receiving weekly bolus 5-FU plus FA (500 mg/m^2), in a NSABP trial suffered NCI grade 3 toxicity (Wolmark et al., 1996). Clearly, toxicity is a major cost of 5-FU/FA therapy, measured both in patient suffering and in financial terms (the cost of care for drug induced illness).

Other Factors

Many non-genetic factors influence the response of cancers to drugs, including tumor location, vasculature, cell growth fraction and various drug resistance mechanisms. It will therefore not be possible to explain all heterogeneity in response to 5-FU/FA by genetic variation. However, based on genetic studies of other quantitative traits it seems likely that a significant fraction of variation in drug response can be explained (see below).

XII. 2.1.2 Metabolic Pathways that Affect 5-FU/FA Action

The biochemical pathways of 5-FU metabolism have been studied extensively. Likewise, folate metabolism has been well investigated and the enzymes that form and consume 5, 10-methylenetetrahydrofolate are well known. The principal metabolic pathways that influence the pharmacologic action of 5-FU are summarized in Figure 1.

Figure 1. 5-FU metabolism and inhibition of thymidylate formation. Enzymes: 1. uridine phosphorylase; 2. thymidine phosphorylase; 3. orotate phosphoribosyl transferase; 4. thymidine kinase; 5. uridine kinase; 6. ribonucleotide reductase; 7. thymidylate synthase; 8. dCMP deaminase; 9. nucleoside monophosphate kinase; 10. nucleoside diphosphate kinase; 11. nucleoside diphosphatase or cytidylate kinase; 12: thymine phosphorylase. FH2 = dihydrofolate, FH4 = tetrahydrofolate. The Figure is adapted from Goodman & Gilman's The Pharmacological Basis of Therapeutics, ninth edition, McGraw Hill, 1996, p. 1249.

De novo and salvage routes of pyrimidine nucleotide formation (5-FU anabolism) and inhibition of thymidylate synthase

5-FU is a biologically inactive pyrimidine analog, which must be phosphorylated, and ribosylated to the nucleoside analog fluorodeoxyuridine monophosphate (FdUMP) to have clinical activity. FdUMP formation can occur via several routes, summarized in Figure 1. 5-FU may be converted by uridine phosphorylase to fluorouridine (FUdR; the reverse reaction is catalyzed by uridine nucleosidase) and then to fluorouridine monophosphate (FUMP) by uridine kinase, or FUMP may be formed from 5-FU in one step via transfer of a phosphoribosyl group from 5-phosphoribosyl-1-pyrophosphate (PRPP), catalyzed by orotate phosphoribosyl transferase. FUMP can be converted to FUDP and subsequently FUTP by a nucleoside monophosphate kinase and nucleoside diphosphate kinase, respectively. FUTP is incorporated into RNA by RNA polymerases, which may account in part for 5-FU toxicity as a result of effects on processing or function (e.g. translation). Alternatively, FUDP may be reduced to the dinucleotide level, FdUDP (fluorodeoxyuridine diphosphate) by ribonucleotide diphosphate reductase, a heterodimeric enzyme. FdUDP can then be converted to FdUTP by nucleoside diphosphate kinase and incorporated into DNA by DNA polymerases, which may account for some 5-FU toxicity. Fluoropyrimidine modified DNA may also be targeted by the nucleotide excision repair process. The more important path of FdUDP metabolism with respect to anticancer effects, however, is believed to be conversion to FdUMP by nucleoside diphosphatase (or cytidylate kinase,

a bi-directional enzyme). dUMP is the precursor of dTMP in de novo pyrimidine biosynthesis, a reaction catalyzed by thymidylate synthase and which consumes 5,10-methylenetetrahydrofolate, producing 7,8 dihydrofolate. FdUMP, however, forms an inhibitory (probably covalent) complex with thymidylate synthase in the presence of 5,10-methylenetetrahydrofolate, thereby blocking formation of thymidylate (other than by the salvage pathway via thymidine kinase). The complex anabolism of FdUMP can be simplified by giving the deoxyribonucleoside of 5-FU, 5-fluorodeoxyuridine (also called floxuridine; FUdR), which can be converted to FdUMP in one step by thymidine kinase. However, FUdR is also rapidly converted back to 5-FU by the bi-directional enzyme thymidine phosphorylase.

5-FU catabolism.

Metabolic elimination of 5-FU occurs via a three-step pathway leading to • -alanine. The first and rate limiting enzyme in the elimination pathway is dihydropyrimidine dehydrogenase (DPD), which transforms more than 80% of a dose of 5-FU to the inactive dihydrofluorouracil form. Subsequently dihydropyrimidinase catalyzes opening of the pyrimidine ring to form 5-fluoro-• -ureidopropionate and then • -ureidopropionase (also called • -alanine synthase) catalyzes formation of 2-fluoro-• -alanine. The first two reactions are reversible. The distribution of activity of these enzymes in human populations has not been established, however, a recent population survey of urinary pyrimidine levels in 1,133 adults revealed that levels of dihydrouracil range from 0 - 59 uM/g of creatinine, while uracil levels ranged from 0 - 130 uM/g creatinine (Hayashi et al., 1996), suggesting variation in the activity of enzymes of pyrimidine metabolism. It is worth noting that in animal studies catabolites of 5-FU apparently account for some fraction of 5-FU toxicity (Davis et al., 1994; Spector et al., 1995). This result is the rationale for current human trials of 5-FU combined with DPD inhibitors: if the 5-fluoro- metabolites are responsible for toxicity, then blocking their formation by inhibition of DPD, while simultaneously decreasing 5-FU dosage to compensate for the block in catabolism and excretion, should result in a better therapeutic index.

Folinic acid conversion to tetrahydrofolate.

The conversion of FA to 5,10MTHF can occur via several routes, illustrated in Figure 2

Figure 2. Folate metabolism and formation of 5,10-methylenetetrahydrofolate.

Enzymes: 1. Formimino-tetrahydrofolate cyclodeaminase; 2. methenyltetrahydrofolate synthetase; 3. methenyltetrahydrofolate cyclohydrolase; 4. formyltetrahydrofolate synthetase; 5. formyltetrahydrofolate hydrolase; 6. formyltetrahydrofolate dehydrogenase; 7. methylenetetrahydrofolate dehydrogenase; 8. methylenetetrahydrofolate reductase (MTHFR); 9. homocysteine methyltransferase (also called methionine synthetase); 10. serine transhydroxymethylase; 11. glycine cleavage system; 12. thymidylate synthase; 13. dihydrofolate reductase. Abbreviations: THF = tetrahydrofolate; DHF = dihydrofolate. Note that THF appears twice (i.e. the product of step 6 is also substrate for enzymes 10 and 11. Step 12 also appears in Figure 1, above. This Figure is adapted from Mathews & van Holde, Biochemistry, The Benjamin/Cummings Publishing Co., Redwood City CA, 1990, page 697.

Intracellular reduced folate levels can potentiate 5-FU action by increasing 5,10-methylenetetrahydrofolate levels (5,10-methyleneTHF; see center of Figure 2), thereby stabilizing the ternary inhibitory complex formed with thymidylate synthase and FdUMP. This is the basis for therapeutic modulation of 5-FU with FA. As can be seen in Figure 2, conversion of folinic acid (5-formylTHF) to 5,10-methenylTHF, the precursor of 5,10-methyleneTHF, requires methenyltetrahydrofolate synthetase (enzyme 2 in the Figure). Also, levels of 5,10-methyleneTHF may be affected directly by the activity of methylenetetrahydrofolate dehydrogenase, methylenetetrahydrofolate reductase, serine transhydroxymethylase and the glycine cleavage system enzymes (7, 8, 10 and 11 in Fig. 2), and indirectly by the other enzymes shown in the Figure.

Cell uptake of pyrimidine nucleosides and folinic acid

Human cells have five concentrative nucleoside transporters with varying patterns of tissue distribution (see review by Wang et al., 1997). Two transporters, one with preference for purines and one for pyrimidines have been cloned recently (Felipe et al., 1998). 5-FU entry into cells may be modulated by activity of these transporters, particularly the pyrimidine transporter, although one prospective randomized clinical trial in which the nucleoside transport inhibitor dipyridamole was paired with 5-FU and FA failed to show a difference in outcome compared to 5-FU/FA alone (Kohne et al., 1995). Several folate transport systems have been identified in human cells. Folate receptor 1 (FR1) is a high affinity (nanomolar range) receptor for reduced folates. Three restriction fragment length polymorphisms (RFLPs) have been reported at the FR1 locus (Campbell et al., 1991). Reduced folates are also transported by folate receptor gamma and by a low affinity (1 uM) folate transporter. 15-fold variations in levels of

folate transporter have been described in unselected tumor cell lines (Moscow et al., 1997).

XIII. 2.1.3 Genetically Determined Variation in Response to 5-FU: Studies of Dihydropyrimidine Dehydrogenase Deficiency

Dihydropyrimidine Dehydrogenase Deficiency is Associated with 5-FU Toxicity

5-FU is inactivated by the same metabolic pathway as thymine and uracil (see above). DPD catalyzes the first, rate-limiting step in pyrimidine catabolism and accounts for elimination of most 5-FU. Normal individuals eliminate 5-FU with a half-life of ~10-15 minutes and excrete only 10% of a dose unchanged in the urine. In contrast, people genetically deficient in DPD eliminate 5-FU with a half-life of ~2.5 hours and excrete 90% of a dose unchanged in the urine (Diasio et al., 1988). DPD deficiency has two clinical presentations: (i) an inborn error of metabolism causing some degree of neurologic dysfunction or (ii) asymptomatic until revealed by exposure to 5-FU or other pyrimidine analogs. With either presentation there is combined hyperuraciluria and hyperthyminuria. The vastly increased 5-FU half-life in DPD deficient individuals causes severe toxicity and even death. Recently several mutations have been identified in DPD genes of deficient individuals (Wei et al., 1996), however none of these alleles appears to occur at appreciable frequency, so the cause of wide population variation in DPD levels is still not understood.

Population Studies of DPD Activity Show Wide Variation

Population surveys of DPD activity in normal individuals have been performed using blood and liver samples. These studies reveal a broad unimodal Gaussian distribution of DPD activity over a 7 to 14 fold range, with some individuals having very low or even undetectable levels. For example Etienne et al. (1994) report DPD activity ranging from .065 to .559 nM/min/mg protein in a study of 152 men and 33 women, while Fleming et al. (1993) found DPD activity in 66 cancer patients varied from .17 to .77 nM/min/mg protein. Lu et al (1995) found 18-fold variation in liver DPD assayed in 138 individuals. Milano and Etienne (1994) suggested that the frequency of heterozygous and homozygous deficiency is 3% and .1%, respectively. The DNA sequence alterations responsible for null DPD alleles do not account for the high population variability (Ridge et al., 1997).

DPD Levels are correlated with Response to 5-FU

Intratumoral DPD levels have been measured in patients receiving 5-FU chemotherapy. When complete responders were compared to partial or non-responders, DPD levels were lower in the compete responders (Etienne et al., 1995). Leukocyte DPD levels has also been measured in patients receiving 5-FU/FA chemotherapy. When patients were divided into 3 groups: high, medium and low DPD activity, the frequency of serious side effects was highest in the low DPD group and vice versa (Katona et al., 1997).

XIV. 2.1.4 Variances in Genes That May Affect 5-FU/FA Action

Variagenics has already surveyed thymidylate synthase, ribonucleotide reductase (M1 subunit only), and dihydrofolate reductase and dihydropyrimidine dehydrogenase cDNAs for genetic variation. 36 unrelated individuals were screened using 6 SSCP conditions and DNA sequencing. Other investigators have identified variances in MTHFR, methionine synthase and folate receptor. These findings are summarized in Appendix I.

XV.

XVI. 2.1.5 Analysis of Haplotypes Increases Power of Genetic Analysis

It is evident from work to date that, while DPD activity is weakly predictive of 5-FU toxicity and drug response, there must be other factors that account for some of the variation in patient response. This is to be expected as drug response phenotypes usually vary continuously, and such (quantitative) traits are typically influenced by a number of genes (Falconer and Mackay, 1997). Although it is impossible to determine *a priori* the number of genes influencing a quantitative trait, often only a few loci have large effects, where a large effect is 5-20% of total variation in the phenotype (Mackay, 1995).

Having identified genetic variation in enzymes that may affect 5-FU action, how can we most efficiently address its relation to phenotypic variation? The sequential testing for correlation between phenotypes of interest and single nucleotide polymorphisms may be adequate to detect associations if there are major effects associated with single nucleotide changes; certainly it is worth performing this type of analysis. However there is no way to know in advance whether there are major phenotypic effects associated with single nucleotide changes and, even if there are, there is no way to be sure that the salient variance has been identified by screening cDNAs. A more

powerful way to address the question of genotype-phenotype correlation is to assort genotypes into haplotypes. (A haplotype is the cis arrangement of polymorphic nucleotides on a particular chromosome.) Haplotype analysis has several advantages compared to the serial analysis of individual polymorphisms at a locus with multiple polymorphic sites.

(1) Of all the possible haplotypes at a locus (2^n haplotypes are theoretically possible at a locus with n binary polymorphic sites) only a small fraction will generally occur at a significant frequency in human populations. Thus, association studies of haplotypes and phenotypes will involve testing fewer hypotheses. As a result there is a smaller probability of Type I errors, that is, false inferences that a particular variant is associated with a given phenotype.

(2) The biological effect of each variance at a locus may be different both in magnitude and direction. For example, a polymorphism in the 5' UTR may affect translational efficiency, a coding sequence polymorphism may affect protein activity, a polymorphism in the 3' UTR may affect mRNA folding and half life, and so on. Further, there may be interactions between variances: two neighboring polymorphic amino acids in the same domain - say cys/arg at residue 29 and met/val at residue 166 - may, when combined in one sequence, for example, 29cys-166val, have a deleterious effect, whereas 29cys-166met, 29arg-166met and 29arg-166val proteins may be nearly equal in activity. Haplotype analysis is the best method for assessing the interaction of variances at a locus.

(3) Templeton and colleagues have developed powerful methods for assorting haplotypes and analyzing haplotype/phenotype associations (Templeton et al., 1987). Alleles, which share common ancestry, are arranged into a tree structure (cladogram) according to their time of origin in a population. Haplotypes that are evolutionarily ancient will be at the center of the branching structure and new ones (reflecting recent mutations) will be represented at the periphery, with the links representing intermediate steps in evolution. The cladogram defines which haplotype-phenotype association tests should be performed to most efficiently exploit the available degrees of freedom, focusing attention on those comparisons most likely to define functionally different haplotypes (Haviland et al., 1995). This type of analysis has been used to define interactions between heart disease and the apolipoprotein gene cluster (Haviland et al 1995) and Alzheimer's Disease and the Apo-E locus (Templeton 1995) among other studies, using populations as small as 50 to 100 individuals.

XVII. 2.1.6 Biochemical Studies of Alternate Allelic Forms of DPD

The power of genetic analysis can be augmented by biochemical studies of alternate allelic forms of enzymes. Biochemical data on the distribution of activity of a series of enzymes in a biochemical pathway provides the basis for metabolic flux analysis (Keightly, 1996). It is beyond the scope of this clinical trial to analyze biochemical variation in the enzymes of pyrimidine and folate metabolism. However, since Variagenics has identified new variances in DPD that may plausibly affect enzyme expression or activity, and because DPD is already proven to play a role in 5-FU response, parallel studies will be conducted to investigate the relationship between genotype and biochemistry for this enzyme.

DPD cDNAs have been cloned from a variety of higher eukaryotes and binding sites for its cofactors, prosthetic groups and substrate have been defined experimentally or by analogy with known consensus motifs (Yokata et al., 1994). The DPD polymorphisms that affect protein sequence occur at amino acids 29 (cys/arg) and 166 (met/val) in the amino-terminal one-third of the protein. Phylogenetic comparison of this region from boar, human, cow, fly, and bacteria (see below) shows that there are actually two highly conserved motifs that resemble either iron/sulfur or zinc binding motifs, the latter being more likely due to the spacing of the cysteine residues. The region around the met/val polymorphism at amino acid 166 is highly conserved. Even the spacing of the putative zinc-finger domains is maintained between distantly related species, hinting at their importance. Since amino acid 166 is close to a highly conserved (and probably functionally important) region and is itself conserved, being a methionine in all species, it seems likely that perturbations in this position would have consequence. The polymorphism substitutes a long amino acid side chain capable of hydrogen bonding (methionine) for a compact, hydrophobic amino acid (valine). The region around amino acid 29 is not as well conserved.

XVIII. 2.2 Study Rationale

5-fluorouracil (5-FU) is a fluorinated pyrimidine analog that is widely used in chemotherapy. The effectiveness of 5-FU is potentiated by folinic acid (FA: generic name: leukovorin). The combination of 5-FU and FA is standard therapy for stage III/IV colon cancer. Patient responses to 5-FU and 5-FU/FA vary widely, ranging from complete remission of cancer to severe toxicity.

Pyrimidine base analogs are degraded by the same enzymes that degrade endogenous uracil and thymine. Dihydropyrimidine dehydrogenase (DPD) is the first degradative enzyme in this pathway, accounting for catabolism of more than 80% of an administered dose of 5-FU.

Total DPD deficiency (familial pyrimidinemia and pyridinuria) is a rare syndrome associated with 5-FU induced toxicity. A milder defect in DPD activity appears to account for the severe side effects that occur in 1%-3% of unselected cancer patients (Milano and Etienne, 1994).

The major toxic manifestations of 5-FU and FA depend on the schedule of administration and occur mainly in rapidly dividing tissues such as bone marrow and the mucosal lining of the gastrointestinal tract.

This study is designed to test whether genetically encoded biochemical variations in the enzymes of pyrimidine catabolism, nucleotide metabolism and folic acid metabolism, among patients treated with a weekly or monthly schedule of 5-FU+FA, account for some of the variation in drug toxicity. Applications of a successful pharmacogenetic study lie in the direction of safer, more efficacious, and hence more economical use of 5-FU, guided by genetic tests.

XIX. 3. OBJECTIVES

XX. 3.1 Primary Objective

The primary objective of this study is to compare the variance frequency distribution in the dihydropyrimidine dehydrogenase (DPD) gene between two groups of patients with solid tumors, treated by weekly or monthly regimen of 5-FU+FA and defined by level of toxicity (graded according to the NCI common toxicity criteria) as:

- Group 1: patients with high toxicity (grade III / IV on NCI criteria)
- Group 2: patients with minimal toxicity (grade 0 / I / II on NCI criteria)

XXI. 3.2 Secondary Objectives

The secondary objectives of the study are to determine the DPD gene haplotype

frequency distribution and the variance and/or haplotype frequency distributions in selected genes (other than DPD gene –see Appendix I-) between two groups of patients with solid tumors, treated by weekly or monthly regimen of 5-FU+FA and defined by level of toxicity. Analyses will be done globally, then by regimen (monthly vs. weekly) and by type of toxicity (gastrointestinal vs. bone marrow).

XXII. 4. STUDY DESIGN

XXIII. 4.1 Study Outline

The study will be done at *selected medical institution.*

The study is a single-center, case-control study. The duration of the study is expected to be not more than 8 months.

Genetic analysis of anonymized patient samples will take place at the *study sponsor.*

XXIV. 4.2 Subject Withdrawal from the Study

Subjects who desire to discontinue participation in this study must be withdrawn from the study.

XXV. 4.3 Discontinuation of the Study

This study may be terminated by *the study sponsor*, after consultation with the Advisory Committee (see Section 11.2), at any time.

XXVI. 5. STUDY POPULATION

XXVII. 5.1 Number of Subjects

Ninety (90) subjects will be recruited for the study.

XXVIII. 5.2 Inclusion Criteria

To be eligible for entry into this study, candidates must meet the following eligibility criteria at the time of enrollment:

1. Above age of 18 years.

2. Diagnosis of solid tumor.

3. Treatment with a weekly or monthly regimen of 5-fluorouracil (5-FU) plus folinic acid (FA)

4. Classified according to the NCI common toxicity criteria as 0, I, II, III or IV grade.

5. Give written informed consent prior to any testing under this protocol, including screening tests and evaluations that are not considered part of the subject's routine care.

XXIX. 5.3 Exclusion Criteria

Candidates will be excluded from study entry if any of the following exclusion criteria exist at the time of enrollment:

Medical History

1. Diagnosis of cancer other than solid tumor.

2. Classified according to the NCI common toxicity criteria as grade II.

3. Known history of HIV, HBV or Hepatitis C virus infection (undesirable for making permanent cell line).

Treatment History

4. Treatment with 5-FU + FA but with other schedule than weekly or monthly.

5. Concomitant treatment with other cancer drugs than 5-FU+FA.

Miscellaneous

6. Unwillingness or inability to comply with the requirements of this protocol.

XXX. 5.4 Screening Log

For every patient initially considered for inclusion in this study, it is required to document and to specifically state the reason(s) for their exclusion.

XXXI. 6. ALLOCATION PROCEDURE

When the eligibility review screening has been completed and the subject has been found eligible for admission to the study, the subject will be assigned to one of the two following group, depending on the 5-FU+FA related toxicity he has experienced in the past:

- Group 1: patients with high toxicity (grade III / IV on NCI criteria)
- Group 2: patients with minimal toxicity (grade 0 / I / II on NCI criteria)

7. SCHEDULE OF EVENTS

XXXII. Patients

Patients will only be required to come for giving informed consent, then having one blood drawing (17ml total) –see Appendix II-.

Study Personnel

The following personnel will be involved in the conduct of this study.

- A *treating physician* who will oversee subject assignment and discuss the protocol with the subject in order to obtain informed consent.
- A *treating nurse* who will assist the treating physician in subject identification management and perform blood sampling.
- A *data manager* who will collect and enter data in the clinical database.

Tests and Evaluations

The tests and evaluations described below must be performed *by the required study personnel* in order to determine subject eligibility.

Treating physician

- Chart and demographic (sex, age, etc) reporting, inclusion/exclusion criteria checking.

Treating nurse

- Blood sampling

Data manager

- Clinical data entry.

XXXIII. 11. STATISTICAL STATEMENT AND ANALYTICAL PLAN

XXXIV. 11.1 Sample Size Considerations

The primary endpoint of this study is to measure and compare genotype distributions of the DPD gene in patients with and without 5-FU+FA toxicity. In order to be able to make a sample size calculation, we will ignore the complexities of the underlying genetic model and treat the data as *n* independent ordinary 2 x 2 contingency tables for the *n* variances in the cases and controls. So, using the 2 most frequent DPD variances listed in Appendix 1 and an odds-ratio of 4.00 for cases vs. controls, we can determine the sample size for every variance, with an equal number of subjects in each phenotypic (i.e. toxicity) group, required to detect, with 80% power at a two-sided significance level of 0.05, a statistically significant difference between distributions:

- nucleotide 3925: 44 patients per group
- nucleotide 3937: 43 patients per group.

A total of 90 patients (45 per group) will so be recruited.

11.2 Description of Objectives and EndpointS

XXXV. 11.2.1 Primary Objective and Endpoints

The primary objective of this study is to compare the variance frequency distributions in the dihydropyrimidine dehydrogenase (DPD) gene between two groups of patients with solid tumors, treated by weekly or monthly regimen of 5-FU+FA and defined by level of toxicity (grade 0/I/II vs. grade III/IV).

XXXVI. 11.2.2 Secondary Objectives and Endpoints

The secondary objectives of the study are:

1. To determine which DPD gene variance(s) is(are) associated to 5-FU+FA toxicity

2. To determine which DPD haplotype(s) is(are) associated to 5-FU+FA toxicity.

3. To determine if one or more of the other gene variances (see Appendix 1) is(are) associated to 5-FU+FA toxicity

4. To determine if one or more of the other haplotypes is(are) associated to 5-FU+FA toxicity.

11.3 CRiteria for the Endpoints

Since we do not know the mode of inheritance of a potential toxic susceptibility, we will ignore in a first step the complexities of the underlying genetic model and treat the data as an ordinary *n x 2* contingency table for the *n* variances in the cases and controls. Then, for every variance, we will compare genotype frequencies in order to detect a potential effect of homo- vs. heterozygosity.

We will also compare haplotype frequencies of *r* predetermined haplotypes. The method of cladograms (Templeton et al., 1987) will be used in an attempt to find out the smallest possible number *r*. In this method the evolutionary relationships between present day haplotypes are represented as a tree or cladogram.

XXXVII. 11.4 Statistical Methods To Be Used in Objective Analyses

The statistical significance of the difference between variance frequencies will be assessed by a Pearson chi-squared test of homogeneity of proportions with *n-1* degrees of freedom. Then, in order to determine which variance(s) is(are) responsible for an eventual significance, we will consider each variance individually against the rest, yielding up to *n* comparisons each based on a 2 x 2 table. This should result in chi-squared tests that are individually valid but taking the most significant of these tests is a form of multiple testing. A Bonferroni's adjustment for multiple testing will so be made to the P-values such as $p^* = 1-(1-p)^n$.

The statistical significance of the difference between genotype frequencies associated to every variance will be assessed by a Pearson chi-squared test of homogeneity of proportions with 2 degrees of freedom, using the same Bonferroni's adjustment as above.

Testing for unequal haplotype frequencies between cases and controls can be considered in the same framework as testing for unequal variance frequencies since a single variance can be considered as a haplotype of a single locus. The relevant likelihood ratio test compares a model where two separate sets of haplotype frequencies apply to the cases and controls, to one where the entire sample is characterized by a single common set of haplotype frequencies. This can be performed by repeated use of a computer program (Terwilliger and Ott, 1994) to successively obtain the log-likelihood corresponding to the set of haplotype frequency estimates on the cases (ln L_{case}), on the controls (ln $L_{control}$) and on the overall (ln $L_{combined}$). The test statistic 2(ln L_{case} + ln $L_{control}$ - ln $L_{combined}$) is then a chi-squared with $r-1$ degrees of freedom (where r is the number of haplotypes).

To test for potential confounding effects or effect-modifiers, such as sex, age, etc. logistic regression will be used with case-control status as the outcome variable, and genotypes and covariates (plus possible interactions) as predictor variables.

XXXVIII. 12. ETHICAL REQUIREMENTS

XXXIX. 12.1 Declaration of Helsinki

See Appendix III.

XL. 12.2 Subject Information and Consent

Prior to any testing under this protocol, including screening tests and evaluations, written informed consent must be obtained from the subject in accordance with the Standards of the Partners Cancercare Human Protection Committee (HPC).

The background of the proposed study and the benefits and risks of the procedures and study will be explained to the subject. A copy of the informed consent document signed and dated by the subject must be given to the subject. Confirmation of a subject's informed consent must also be documented in the subject's medical records prior to any testing under this protocol, including screening tests and evaluations.

XLI. 12.3 Subject Data Protection

The subject will not be identified by name or other any identifying characteristic in any study reports, and these reports will be used for research purposes only.*the study sponsor*, its designee(s), and various Government Health Agencies may inspect the records of this study. All relevant demographic and historical data regarding patient drug response will be recorded in an anonymized database.

XLII. 13. FURTHER REQUIREMENTS AND GENERAL INFORMATION

XLIII. 13.1 Study Committee

Advisory Committee

An Advisory Committee will be formed to provide scientific and medical direction for the study and to oversee the administrative progress of the study. The Advisory Committee will meet at least once a month to monitor subjects. The Advisory Committee will determine whether the study should be stopped or amended for any reason.

The Advisory Committee will be comprised of the Director of Clinical Pharmacogenetics, Vice-President for Discovery Research from *the study sponsor*

(and/or their designee) and participating investigators. The principal investigator will chair the Advisory Committee.

XLIV. 13.2 Changes to Final Study Protocol

All protocol amendments must be submitted to the IRB/REB/EC. Protocol modifications that impact on subject safety, the scope of the investigation, or affect the scientific quality of the study must be approved by the IRB/REB/EC and submitted to the appropriate regulatory authorities before initiation. However, Variagenics may, at any time, amend this protocol to eliminate an apparent immediate hazard to a subject. In this case, the appropriate regulatory authorities will be subsequently notified. In the event of a protocol modification, the subject consent form may require similar modifications.

XLV. 13.3 Record Retention

The Principal Investigator must maintain the records of signed consent forms, CRFs, all correspondences, dates of any monitoring visits, and records that support this information for a period of 15 years following notification by *the study sponsor* that the clinical investigations have been completed or discontinued. All local laws regarding retention of records must also be followed.

XLVI. 13.4 Reporting and Communication of Results

All information concerning *the study sponsor's* perations, such as patent applications, formulas, manufacturing processes, basic scientific data, and formulation information supplied by *the study sponsor* and not published previously, are considered confidential and shall remain the sole property of the *study sponsor*. The investigator agrees to use this information only in conducting this study and shall not use it for any other purposes without *the study sponsor's* written approval. The investigator agrees not to disclose *the study sponsor's* confidential information to anyone except to people involved in the study who need such information to assist in conducting the study and then only on like terms of confidentiality and nonuse.

It is understood by the investigator that the information developed from this clinical study will be used by *the study sponsor* and therefore may be disclosed as required to

other clinical investigators, to the U.S. Food and Drug Administration, the Canadian Health and Welfare Health Protection Branch, the European Medicines Evaluation Agency, and to other government agencies. In order to allow for the use of the information derived from the clinical studies, it is understood that there is an obligation to provide *the study sponsor* with complete test results and all data developed in the study.

No publication or disclosure of study results will be permitted except as specified in a separate, written agreement between *the study sponsor* and the investigator.

XLVII. 13.5 PROTOCOL COMPLETION

The IRB/REB/EC must be notified of completion or termination of the protocol. Within 3 months of protocol completion or termination, the investigator must provide a final clinical summary report to the IRB/REB/EC. The Principal Investigator must maintain an accurate and complete record of all submissions made to the IRB/REB/EC, including a list of all reports and documents submitted. A copy of these reports should be sent to *the study sponsor*.

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XLIX. SIGNED AGREEMENT OF THE STUDY PROTOCOL

I have read the foregoing protocol, VRG-9801, "Case-control study to determine the relationship between toxicity of 5-fluorouracil (5-FU) given with

folinic acid (FA) to patients with solid tumors and DNA sequence variances in enzymes that mediate the action of 5-FU and FA", Version 1, and agree to conduct the study as detailed herein and to inform all who assist me in the conduct of this study of their responsibilities and obligations.

5

10

_
Principal Investigator's Signature Date

15 _
Principal Investigator's Name (Print)

_
20 Investigational Site (Print)

APPENDIX II

L. Procedures for handling blood samples for cell line establishment

This document describes procedures for handling blood samples from cancer patients enrolled in trial for genetic studies at *the study sponsor*. The approach will be to first establish permanent lymphoblastoid cell lines. DNA and RNA will subsequently be extracted from these cell lines. This procedure will save the effort of purifying DNA and RNA directly from blood. Since the pharmacogenetic hypotheses to be investigated relate to the effect of genotype, not mRNA expression levels, lymphoblastoid cell lines should be satisfactory sources of nucleic acid for the genetic studies.

1. Cell line establishment will be done by the *study site institutions* (e.g., Genomics Core Facility of the Massachusetts General Hospital (MGH) Molecular Neurogenetics Unit).

2. From each patient collect two 8.5 ml yellow topped tubes (containing ACD solution A) for lymphoblastoid cell line development. All DNA and RNA will be produced from the cell lines at a later date; therefore there is no need for additional blood drawing.

3. Fill out a DNA/Cell Line Order Sheet. An example is attached. Please note that the patient's name should be anonymized at this point. (The Genomics Core Facility will accept anonymized order forms.) All samples (including those for PK studies) should be assigned the same arbitrary number to allow subsequent matching of clinical, pharmacokinetic and genetic data. Also, the date and time of blood drawing should be marker. DOB should be recorded as month and year only, and sex should be recorded. Record the number of tubes of blood drawn (2), date of draw and date of shipment. Under "Requisition" check off "Transformation only".

4. Arrange for the two ACD blood samples to be delivered to *designated individual at the study site institution* at the address given below:

Name and address of designated individual at study site institution.

Since the blood samples are typically aged at room temperature for a day or two before cell line establishment, it is not urgent that blood be delivered the same day it is drawn. Storage overnight, if necessary, should be at room temperature.

5. Please fax to *the study sponsor* a copy of the cell line order form so we are aware of accumulating cell lines. The fax number is 588-5399. Please fax to the attention of *the designated individual for the study sponsor.*

6. Once cell lines are established, vials will be archived at the *study site institution*, where they will be available to investigators.

7. Questions for *the study sponsor* should be addressed to *the designated individual.*

Example 11

Hardy-Weinberg equilibrium

Evolution is the process of change and diversification of organisms through time, and evolutionary change affects morphology, physiology and reproduction of organisms, including humans. These evolutionary changes are the result of changes in the underlying genetic or hereditary material. Evolutionary changes in a group of interbreeding individuals or Mendelian population, or simply populations, are described in terms of changes in the frequency of genotypes and their constituent alleles. Genotype frequencies for any given generation is the result of the mating among members (genotypes) of their previous generation. Thus, the expected proportion of genotypes from a random union of individuals in a given population is essential for describing the total genetic variation for a population of any species. For example, the expected number of genotypes that could form from the random union of two alleles, A and a, of a gene are AA, Aa and aa. The expected frequency of genotypes in a large, random mating population was discovered to remain constant from generation to generation; or achieve Hardy-Weinberg equilibrium, named after its discoverers. The expected genotypic frequencies of alleles A and a (AA, 2Aa, aa) are conventionally described in terms of $p^2 + 2pq + q^2$ in which p and q are the allele frequencies of A and a. In this equation ($p^2 + 2pq + q^2 = 1$), p is defined as the frequency of one allele and q

as the frequency of another allele for a trait controlled by a pair of alleles (A and a). In other words, p equals all of the alleles in individuals who are homozygous dominant (AA) and half of the alleles in individuals who are heterozygous (Aa) for this trait. In mathematical terms, this is

$$p = AA + \tfrac{1}{2}Aa$$

Likewise, q equals the other half of the alleles for the trait in the population, or

$$q = aa + \tfrac{1}{2}Aa$$

Because there are only two alleles in this case, the frequency of one plus the frequency of the other must equal 100%, which is to say

$$p + q = 1$$

Alternatively,

$$p = 1 - q \text{ OR } q = 1 - p$$

All possible combinations of two alleles can be expressed as:

$$(p + q)^2 = 1$$

or more simply,

$$p^2 + 2pq + q^2 = 1$$

In this equation, if p is assumed to be dominant, then p^2 is the frequency of homozygous dominant (AA) individuals in a population, 2pq is the frequency of heterozygous (Aa) individuals, and q^2 is the frequency of homozygous recessive (aa) individuals.

From observations of phenotypes, it is usually only possible to know the frequency of homozygous dominant or recessive individuals, because both dominant and recessives will express the distinguishable traits. However, the Hardy-Weinberg equation allows us to determine the expected frequencies of all the genotypes, if only p or q is known. Knowing p and q, it is a simple matter to plug these values into the Hardy-Weinberg equation ($p^2 + 2pq + q^2 = 1$). This then provides the frequencies of all three genotypes for the selected trait within the population.

This illustration shows Hardy-Weinberg frequency distributions for the genotypes AA, Aa, and aa at all values for frequencies of the alleles, p and q. It should be noted that the proportion of heterozygotes increases as the values of p and q approach 0.5.

Linkage disequilibirum

Linkage is the tendency of genes or DNA sequences (e.g. SNPs) to be inherited together as a consequence of their physical proximity on a single chromosome. The closer together the markers are, the lower the probability that they will be separated during DNA crossing over, and hence the greater the probability that they will be inherited together. Suppose a mutational event introduces a "new" allele in the close proximity of a gene or an allele. The new allele will tend to be inherited together with the alleles present on the "ancestral," chromosome or haplotype. However, the resulting association, called linkage disequilibrium, will decline over time due to recombination. Linkage disequilibrium has been used to map disease genes. In general, both allele and haplotype frequencies differ among populations. Linkage disequilibrium is varied among the populations, being absent in some and highly significant in others.5

Quantification of the relative risk of observable outcomes of a Pharmacogenetics Trial

Let PlaR be the placebo response rate (0% (PlaR (100%) and TntR be the treatment response rate (0% (TntR (100%) of a classical clinical trial. ObsRR is defined as the relative risk between TntR and PlaR:

ObsRR = TntR / PlaR.

Suppose that in the treatment group there is a polymorphism in relation to drug metabolism such as the treatment response rate is different for each genotypic subgroup of patients. Let q be the allele a frequency of a recessive biallelic locus (e.g. SNP) and p = 1 – q the allele A frequency. Following Hardy-Weinberg equilibrium, the relative frequency of homozygous and heterozygous patients are as follow:

AA: p2 Aa: 2pq aa: q2

with

(p2+2pq+q2) = 1.

Let's define AAR, AaR, aaR as respectively the response rates of the AA, Aa and aa patients. We have the following relationship:

TntR = AAR*p2 + AaR*2pq + aaR*q2.

Suppose that the aa genotypic group of patients has the lowest response rate, i.e. a response rate equal to the placebo response rate (which means that the polymorphism has no impact on natural disease evolution but only on drug action) and let's define

ExpRR as the relative risk between AAR and aaR, as

ExpRR = AAR / aaR.

From the previous equations, we have the following relationships:

ObsRR (ExpRR (1/PlaR

TntR / PlaR = (AAR*p2 + AaR*2pq + aaR*q2) / PlaR

The maximum of the expected relative risk, max(ExpRR), corresponding to the case of heterozygous patients having the same response rate as the placebo rate, is such that:

ObsRR = ExpRR*p2 + 2pq + q2 ⇔ ExpRR = (ObsRR – 2pq –q2) / p2

min(ExpRR),

corresponding to the case of heterozygous patients having the same response rate as the homozygous non-affected patients, is such that:

ObsRR = ExpRR*(p2 + 2pq) +q2 ⇔ ExpRR = (ObsRR –q2) / (p2 + 2pq)

For example, if q = 0.4, PlaR = 40% and ObsRR = 1.5 (i.e. TntR = 60%), then 1.6 (ExpRR (2.4. This means that the best treatment response rate we can expect in a genotypic subgroup of patients in these conditions would be 95.6% instead of 60%.

This can also be expressed in terms of maximum potential gain between the observed difference in response rates (TntR – PlaR) without any pharmacogenetic hypothesis and the maximum expected difference in response rates (max(ExpRR)*PlaR – TntR) with a strong pharmacogenetic hypothesis:

(max(ExpRR)*PlaR – TntR) = [(ObsRR – 2pq –q2) / p2] * PlaR – TntR

⇔ (max(ExpRR)*PlaR – TntR) = [TntR – PlaR*(2pq + q2) –TntR*p2]/p2

⇔ (max(ExpRR)*PlaR – TntR) = [TntR*(1- p2)– PlaR*(2pq + q2)]/p2

⇔ (max(ExpRR)*PlaR – TntR) = [(1 – p2) / p2] * (TntR – PlaR)

that is for the previous example, (95.6% – 60%) = [(1 – 0.62)/0.62]* (60% -40%) = 35.6%

Suppose that, instead of one SNP, we have p loci of SNPs for one gene. This means that we have 2p possible haplotypes for this gene and (2p)(2p-1)/2 possible genotypes. And with 2 genes with p1 and p2 SNP loci, we have [(2p1)(2p1-1)/2]*[(2p2)(2p2-1)/2] possibilities; and so on. Examining haplotypes instead of

combinations of SNPs is especially useful when there is linkage disequilibrium enough to reduce the number of combinations to test, but not complete since in this latest case one SNP would be sufficient. Yet the problem of frequency above still remains with haplotypes instead of SNPs since the frequency of a haplotype cannot be higher than the highest SNP frequency involved.

Statistical Methods to be used in Objective Analyses

The statistical significance of the differences between variance frequencies can be assessed by a Pearson chi-squared test of homogeneity of proportions with *n-1* degrees of freedom. Then, in order to determine whih variance(s) is(are) responsible for an eventual significance, we can consider each variance individually against the rest, up to *n* comparisons, each based on a 2x2 table. This should result in chi-sequared tests that are individually valid, but taking the most significant of these tests is a form of multiple testing. A Bonferroni's adjustment for multiple testing will thus be made to the P-values, such as $p^*=1-(1-p)^n$.

The statistical significance of the difference between genotype frequencies associated to every variance can be assessed by a Pearson chi-squared test of homogeneity of proportions with 2 degrees of freedom, using the same Bonferroni's adjustment as above.

Testing for unequal haplotype frequencies between cases and controls can be considered in the same framework as testing for unequal variance frequencies since a single variance can be considered as a haplotype of a single locus. The relevant likelihood ratio test compares a model where two seqarate sets of haplotype frequencies apply to the cases and controls, to one where the entire sample is characterized by a single common set of haplotype frequencies. This can be performed by repeated use of a computer program (Terwilliger and Ott, 1994, Handbook of Human Linkage Analysis, Baltimore, John Hopkins University Press) to successively obtain the log-likelihood corresponding to the set of haplotpe frequency estimates on the cases ($\ln L_{case}$), on the controls ($\ln L_{control}$), and on the overall ($\ln L_{combined}$). The test statistic $2((\ln L_{case})+ (\ln L_{control})- (\ln L_{combined}))$ is then chi-squared with *r-1* degrees of

freedom (where r is the number of haplotypes).

To test for potentially confounding effects or effect-modifiers, such as sex, age, etc., logistic regression can be used with case-control status as the outcome variable, and genotypes and covariates (plus possible interactions) as predictor variables.

Example 12 Exemplary Pharmacogenetic Analysis Steps

In accordance with the discussion of distribution frequencies for variances, alleles, and haplotypes, variance detection, and correlation of variances or haplotypes with treatment response variability, the points below list major items which will typically be performed in an analysis of the pharmacogenetic determination of the effects of variances in the treatment of a disease and the selection/optimization of treatment.

- List candidate gene/genes for a known genetic disease, and assign them to the respective metabolic pathways.
- Determine their alleles, observed and expected frequencies, and their relative distributions among various ethnic groups, gender, both in the control and in the study (case) groups
- Measure the relevant clinical/phenotypic (biochemical / physiological) variables of the disease
- If the causal variance/allele in the candidate gene is unknown, then determine linkage disequilibria among variances of the candidate gene(s)
- Divide the regions of the candidate genes into regions of high linkage disequilibrium and low disequilibrium
- Develop haplotypes among variances that show strong linkage disequilibrium using the computation methods.
- Determine the presence of rare haplotypes experimentally. Confirm if the computationally determined rare haplotypes agree with the experimentally determined haplotypes. If there is a disagreement between the experimentally

determined haplotypes and the computationally derived haplotypes, drop the computationally derived rare haplotypes,

- Construct cladograms from these haplotypes using the Templeton (1987) algorithm.
- Note regions of high recombination. Divide regions of high recombination further to see patterns of linkage disequilibria.
- Establish association between cladograms and clinical variables using the nested analysis of variance as presented by Templeton (1995), and assign causal variance to a specific haplotype
- For variances in the regions of high recombination, use permutation tests for establishing associations between variances and the phenotypic variables
- If two or more genes are found to affect a clinical variable determine the relative contribution of each of the genes or variances in relation to the clinical variable, using step-wise regression or discriminant function or principal component analysis.
- Determine the relative magnitudes of the effects of any of the two variances on the clinical variable due to their genetic (additive, dominant or epistasis) interaction.
- Using the frequency of an allele or haplotypes, as well as biochemical/clinical variables determined in the *in vitro* or *in vivo* studies, determine the effect of that gene or allele on the expression of the clinical variable, according to the measured genotype approach of Boerwinkle et al (Ann. Hum. Genet 1986).
- Stratify ethnic/ clinical populations based on the presence or absence of a given allele or a haplotype
- Optimize drug dosages based on the frequency of alleles and haplotypes as well as their effects using the measured genotype approach as a guide

Example 13 Method for Producing cDNA

In order to identify sequence variances in a gene by laboratory methods it is in some instances useful to produce cDNA(s) from multiple human subjects. (In other instances it may be preferable to study genomic DNA.). Methods for producing cDNA are known to those skilled in the art, as are methods for amplifying and sequencing the cDNA or portions thereof. An example of a useful cDNA production protocol is

provided below. As recognized by those skilled in the art, other specific protocols can also be used.

cDNA Production

** Make sure that all tubes and pipette tips are RNase-free. (Bake them overnight at 100°C in a vaccum oven to make them RNase-free.)

1 Add the following to a RNase-free 0.2 ml micro-amp tube and mix gently:

24 ul water (DEPC treated)
12 ul RNA (1ug/ul)
12 ul random hexamers(50 ng/ul)

2 Heat the mixture to 70°C for ten minutes.

3 Incubate on ice for 1 minute.

4 Add the following:

16 ul 5 X Synthesis Buffer
8 ul 0.1 M DTT
4 ul 10 mM dNTP mix (10 mM each dNTP)
4 ul SuperScript RT II enzyme

Pipette gently to mix.

5 Incubate at 42°C for 50 minutes.

6 Heat to 70°C for ten minutes to kill the enzyme, then place it on ice.

7 Add 160 ul of water to the reaction so that the final volume is 240 ul.

8 Use PCR to check the quality of the cDNA. Use primer pairs that will give a ~800 base pair long piece. See "PCR Optimization" for the PCR protocol.

The following chart shows the reagent amounts for a 20 ul reaction, a 80 ul reaction, and a batch of 39 (which makes enough mix for 36) reactions:

	20 ul X 1 tube	**80 ul X 1 tube**	**80ul X 39 tubes**	
water	6 ul	24 ul	936	water
RNA	3 ul	12 ul		RNA
random hexamers	3 ul	12 ul	468	random hexamers
synthesis buffer	4 ul	16 ul	624	synthesis buffer
0.1 M DTT	2 ul	8 ul	312	0.1 M DTT

10mM dNTP	1 ul	4 ul	156	10mM dNTP
SSRT	1 ul	4 ul	156	SSRT

Example 14

Method for Detecting Variances by Single Strand Conformation Polymorphism (SSCP) Analysis

This example describes the SSCP technique for identification of sequence variances of genes. SSCP is usually paired with a DNA sequencing method, since the SSCP method does not provide the nucleotide identity of variances. One useful sequencing method, for example, is DNA cycle sequencing of ^{32}P labeled PCR products using the Femtomole DNA cycle sequencing kit from Promega (WI) and the instructions provided with the kit. Fragments are selected for DNA sequencing based on their behavior in the SSCP assay.

Single strand conformation polymorphism screening is a widely used technique for identifying an discriminating DNA fragments which differ from each other by as little as a single nucleotide. As originally developed by Orita et al. (Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci U S A.* 86(8):2766-70, 1989), the technique was used on genomic DNA, however the same group showed that the technique works very well on PCR amplified DNA as well. In the last 10 years the technique has been used in hundreds of published papers, and modifications of the technique have been described in dozens of papers. The enduring popularity of the technique is due to (1) a high degree of sensitivity to single base differences (>90%) (2) a high degree of selectivity, measured as a low frequency of false positives, and (3) technical ease. SSCP is almost always used together with DNA sequencing because SSCP does not directly provide the sequence basis of differential fragment mobility. The basic steps of the SSCP procdure are described below.

When the intent of SSCP screening is to identify a large number of gene variances it is useful to screen a relatively large number of individuals of different racial, ethnic and/or geographic origins. For example, 32 or 48 or 96 individuals is a convenient number to screen because gel electrophoresis apparatus are available with 96

wells (Applied Biosystems Division of Perkin Elmer Corporation), allowing 3 X 32, 2 X 48 or 96 samples to be loaded per gel.

The 32 (or more) individuals screened should be representative of most of the worlds major populations. For example, an equal distribution of Africans, Europeans and Asians constitutes a reasonable screening set. One useful source of cell lines from different populations is the Coriell Cell Repository (Camden, NJ), which sells EBV immortalized lyphoblastoid cells obtained from several thousand subjects, and includes the racial/ethnic/geographic background of cell line donors in its catalog. Alternatively, a panel of cDNAs can be isolated from any specific target population.

SSCP can be used to analyze cDNAs or genomic DNAs. For many genes cDNA analysis is preferable because for many genes the full genomic sequence of the target gene is not available, however, this circumstance will change over the next few years. To produce cDNA requires RNA. Therefore each cell lines is grown to mass culture and RNA is isolated using an acid/phenol protocol, sold in kit form as Trizol by Life Technologies (Gaithersberg, MD). The unfractionated RNA is used to produce cDNA by the action of a modified Maloney Murine Leukemia Virus Reverse Transcriptase, purchased in kit form from Life Technologies (Superscript II kit). The reverse transcriptase is primed with random hexamer primers to initiate cDNA synthesis along the whole length of the RNAs. This proved useful later in obtaining good PCR products from the 5' ends of some genes. Alternatively, oligodT can be used to prime cDNA synthesis.

Material for SSCP analysis can be prepared by PCR amplification of the cDNA in the presence of one α ^{32}P labeled dNTP (usually α ^{32}P dCTP). Usually the concentration of nonradioactive dCTP is dropped from 200 uM (the standard concentration for each of the four dNTPs) to about 100 uM, and ^{32}P dCTP is added to a concentration of about 0.1-0.3 uM. This involves adding a 0.3- 1 ul (3-10 uCi) of ^{32}P cCTP to a 10 ul PCR reaction. Radioactive nucleotides can be purchased from DuPont/New England Nuclear.

The customary practice is to amplify about 200 base pair PCR products for SSCP, however, an alternative approach is to amplify about 0.8-1.4 kb fragments and then use several cocktails of restriction endonucleases to digest those into smaller fragments of about 0.1-0.4kb, aiming to have as many fragments as possible between

.15 and .3 kb. The digestion strategy has the advantage that less PCR is required, reducing both time and costs. Also, several different restriction enzyme digests can be performed on each set of samples (for example 96 cDNAs), and then each of the digests can be run separately on SSCP gels. This redundant method (where each nucleotide is surveyed in three different fragments) reduces both the false negative and false positive rates. For example: a site of variance might lie within 2 bases of the end of a fragment in one digest, and as a result not affect the conformation of that strand; the same variance, in a second or third digest, would likely lie in a location more prone to affect strand folding, and therefore be detected by SSCP.

After digestion, the radiolabelled PCR products are diluted 1:5 by adding formamide load buffer (80% formamide, 1X SSCP gel buffer) and then denatured by heating to 90%C for 10 minutes, and then allowed to renature by quickly chilling on ice. This procedure (both the dilution and the quick chilling) promotes intra- (rather than inter-) strand association and secondary structure formation. The secondary structure of the single strands influences their mobility on nondenaturing gels, presumably by influencing the number of collisions between the molecule and the gel matrix (i.e., gel sieving). Even single base differences consistently produce changes in intrastrand folding sufficient to register as mobility differences on SSCP.

The single strands were then resolved on two gels, one a 5.5% acrylamide, 0.5X TBE gel, the other an 8% acrylamide, 10% glycerol, 1X TTE gel. (Other gel recipes are known to those skilled in the art.) The use of two gels provides a greater opportunity to recognize mobility differences. Both glycerol and acrylamide concentration have been shown to influence SSCP performance. By routinely analyzing three different digests under two gel conditions (effectively 6 conditions), and by looking at both strands under all 6 conditions, one can achieve a 12-fold sampling of each base pair of cDNA. However, if the goal is to rapidly survey many genes or cDNAs then a less redundant procedure would be optimal.

Example 15

Method for Detecting Variances by T4 endonuclease VII (T4E7) mismatch cleavage method

The enzyme T4 endonuclease VII is derived from the bacteriophage T4. T4 endonuclease VII is used by the bacteriophage to cleave branched DNA intermediates which form during replication so the DNA can be processed and packaged. T4 endonuclease can also recognize and cleave heteroduplex DNA containing single base mismatches as well as deletions and insertions. This activity of the T4 endonuclease VII enzyme can be exploited to detect sequence variances present in the general population.

The following are the major steps involved in identifying sequence variations in a candidate gene by T4 endonuclease VII mismatch cleavage:

1. Amplification by the polymerase chain reaction (PCR) of 400-600 bp regions of the candidate gene from a panel of DNA samples The DNA samples can either be cDNA or genomic DNA and will represent some cross section of the world population.
2. Mixing of a fluorescently labeled probe DNA with the sample DNA. Heating and cooling the mixtures causing heteroduplex formation between the probe DNA and the sample DNA.
3. Addition of T4 endonuclease VII to the heteroduplex DNA samples. T4 endonuclease will recognize and cleave at sequence variance mismatches formed in the heteroduplex DNA.
4. Electrophoresis of the cleaved fragments on an ABI sequencer to determine the site of cleavage.
5. Sequencing of a subset of PCR fragments identified by T4 endonuclease VI to contain variances to establish the specific base variation at that location.

A more detailed description of the procedure is as follows:

A candidate gene sequence is downloaded from an appropriate database. Primers for PCR amplification are designed which will result in the target sequence being divided into amplification products of between 400 and 600 bp. There will be a minimum of a 50 bp of overlap not including the primer sequences between the 5' and

3' ends of adjacent fragments to ensure the detection of variances which are located close to one of the primers.

Optimal PCR conditions for each of the primer pairs is determined experimentally. Parameters including but not limited to annealing temperature, pH, $MgCl_2$ concentration, and KCl concentration will be varied until conditions for optimal PCR amplification are established. The PCR conditions derived for each primer pair is then used to amplify a panel of DNA samples (cDNA or genomic DNA) which is chosen to best represent the various ethnic backgrounds of the world population or some designated subset of that population.

One of the DNA samples is chosen to be used as a probe. The same PCR conditions used to amplify the panel are used to amplify the probe DNA. However, a flourescently labeled nucleotide is included in the deoxy-nucleotide mix so that a percentage of the incorporated nucleotides will be fluorescently labeled.

The labeled probe is mixed with the corresponding PCR products from each of the DNA samples and then heated and cooled rapidly. This allows the formation of heteroduplexes between the probe and the PCR fragments from each of the DNA samples. T4 endonuclease VII is added directly to these reactions and allowed to incubate for 30 min. at 37 C. 10 ul of the Formamide loading buffer is added directly to each of the samples and then denatured by heating and cooling. A portion of each of these samples is electrophoresed on an ABI 377 sequencer. If there is a sequence variance between the probe DNA and the sample DNA a mismatch will be present in the heteroduplex fragment formed. The enzyme T4 endonuclease VII will recognize the mismatch and cleave at the site of the mismatch. This will result in the appearance of two peaks corresponding to the two cleavage products when run on the ABI 377 sequencer.

Fragments identified as containing sequencing variances are subsequently sequenced using conventional methods to establish the exact location and sequence variance.

Example 16

Method for Detecting Variances by DNA sequencing.

Sequencing by the Sanger dideoxy method or the Maxim Gilbert chemical cleavage method is widely used to determine the nucleotide sequence of genes. Presently, a worldwide effort is being put forward to sequence the entire human genome. The Human Genome Project as it is called has already resulted in the identification and sequencing of many new human genes. Sequencing can not only be used to identify new genes, but can also be used to identify variations between individuals in the sequence of those genes.

The following are the major steps involved in identifying sequence variations in a candidate gene by sequencing:

1. Amplification by the polymerase chain reaction (PCR) of 400-700 bp regions of the candidate gene from a panel of DNA samples The DNA samples can either be cDNA or genomic DNA and will represent some cross section of the world population.
2. Sequencing of the resulting PCR fragments using the Sanger dideoxy method. Sequencing reactions are performed using flourescently labeled dideoxy terminators and electrophoresed on an ABI 377 sequencer or its equivalent.
3. Analysis of the resulting data from the ABI 377 sequencer using software programs designed to identify sequence variations between the different samples analyzed.

A more detailed description of the procedure is as follows:

A candidate gene sequence is downloaded from an appropriate database. Primers for PCR amplification are designed which will result in the target sequence being divided into amplification products of between 400 and 700 bp. There will be a minimum of a 50 bp of overlap not including the primer sequences between the 5' and 3' ends of adjacent fragments to ensure the detection of variances which are located close to one of the primers.

Optimal PCR conditions for each of the primer pairs is determined experimentally. Parameters including but not limited to annealing temperature, pH, $MgCl_2$ concentration, and KCl concentration will be varied until conditions for optimal PCR amplification are established. The PCR conditions derived for each primer pair is

then used to amplify a panel of DNA samples (cDNA or genomic DNA) which is chosen to best represent the various ethnic backgrounds of the world population or some designated subset of that population.

PCR reactions are purified using the QIAquick 8 PCR purification kit (Qiagen cat# 28142) to remove nucleotides, proteins and buffers. The PCR reactions are mixed with 5 volumes of Buffer PB and applied to the wells of the QIAquick strips. The liquid is pulled through the strips by applying a vacuum. The wells are then washed two times with 1 ml of buffer PE and allowed to dry for 5 minutes under vacuum. The PCR products are eluted from the strips using 60 ul of elution buffer.

The purified PCR fragments are sequenced in both directions using the Perkin Elmer ABI Prism™ Big Dye™ terminator Cycle Sequencing Ready Reaction Kit (Cat# 4303150). The following sequencing reaction is set up: 8.0 ul Terminator Ready Reaction Mix, 6.0 ul of purified PCR fragment, 20 picomoles of primer, deionized water to 20 ul. The reactions are run through the following cycles 25 times: 96°C for 10 second, annealing temperature for that particular PCR product for 5 seconds, 60°C for 4 minutes.

The above sequencing reactions are ethanol precipitated directly in the PCR plate, washed with 70% ethanol, and brought up in a volume of 6 ul of formamide dye. The reactions are heated to 90°C for 2 minutes and then quickly cooled to 4°C. 1 ul of each sequencing reaction is then loaded and run on an ABI 377 sequencer.

The output for the ABI sequencer appears as a series of peaks where each of the different nucleotides, A, C, G, and T appear as a different color. The nucleotide at each position in the sequence is determined by the most prominent peak at each location. Comparison of each of the sequencing outputs for each sample can be examined using software programs to determine the presence of a variance in the sequence. One example of heterozygote detection using sequencing with dye labeled terminators is described by Kwok *et. al.* (Kwok, P.-Y.; Carlson, C.; Yager, T.D., Ankener, W.,and D. A. Nickerson, *Genomics* 23, 138-144, 1994). The software compares each of the normalized peaks between all the samples base by base and looks for a 40% decrease in peak height and the concomitant appearance of a new peak underneath. Possible variances flagged by the software are further analyzed visually to confirm their validity.

In connection with the provision and description of nucleic acid sequences, the references herein to gene names and to GenBank and OMIM reference numbers provides the relevant sequences, recognizing that the described sequences will, in most cases, also have other corresponding allelic variants. Also, it is recognized that the referenced sequences may contain sequencing error. Such error does not interfere with identification of a relevant gene or portion of a gene, and can be readily corrected by redundant sequencing of the relevant sequence (preferably using both strands of DNA). Nucleic acid molecules or sequences can be readily obtained or determined utilizing the reference sequences. In general, molecules such as nucleic acid hybridization probes and amplification primers can be provided and are described by the selected portion of the reference sequence, corrected if necessary. Thus, nucleic acid hybridization probes and/or primers are thus described by a portion of a reference sequence or a sequence complementary thereto (sequence corrected if necessary), or an allelic variant of such a sequence, which preferably includes at least one variance site, preferably a variance site indicative of the effectiveness of a treatment for a disease or condition, and preferably include at least 12,13,14,15,16,17,18,19,20,23,25,27,30,35,40,45, or 50 nucleotides.

All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods, variances, and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. For example, using other compounds, and/or methods of administration are all within the scope of the present invention. Thus, such

additional embodiments are within the scope of the present invention and the following claims.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Thus, additional embodiments are within the scope of the invention and within the following claims.

Table 10

Variance Table

Hugo	GID	OMIM ID	VGX Symbol	Description	Variance Start	Variance	CDS_Context
U73338	U73338	156570	GEN-69	Methionine Synthase			
					1136G 1252C 1334G 1699T 3150G 3207G 3209G 5444C 5551G 5573C 5659T 5678T 5874C 5934A		
					1136G 1252C 1334G 1699T 3150G 3207G 3209G 5444C 5551G 5573C 5659T 5678T 5874T 5934A		
					1136G 1252C 1334G 1699T 3150A 3207G 3209G 5444C 5551G 5573C 5659T 5678T 5874C 5934A		
					1136G 1252C 1334G 1699T 3150A 3207G 3209G 5444C 5551A 5573C 5659T 5678T 5874C 5934A		
					1136G 1252T 1334G 1699T 3150A 3207G 3209G 5444C 5551G 5573C 5659T 5678T 5874C 5934A		
					1136G 1252C 1334G 1699T 3150A 3207G 3209G 5444A 5551A 5573C 5659T 5678T 5874C 5934A		
						1136	742G>A V248M
						1158	764G>A C255Y
						1252	858C>T Silent
						1334	940G>A D314N
						1699	1305T>C Silent
						3150	2756A>G D919G
						3207	2813G>T S938I
						3209	2815G>C G939R
						5095	4701G>A 3'
						5444	5050C>A 3'
						5551	5157G>A 3'
						5573	5179C>T 3'
						5659	5265T>C 3'
						5678	5284T>C 3'
						5874	5480C>T 3'
						5934	5540A>G 3'
						6750	6356G>A 3'
D78011	D78011	222748	GEN-BO	Dihydropyrimidinase			
					129C 144G 670C 1131G 1158G		
					129T 144G 670C 1131G 1158C		
					129C 144G 670C 1131G 1158C		

129C 144G 670T 1131G 1158C
129 (-1)T>C 5'
144 15G>A Silent
670 541C>T R181W
1131 1002G>A Silent
1158 1029C>G Silent
1479 1350C>G Silent
J03626 J03626 258900 GEN-C6 Uridine monophosphate synthetase (orotate phosphoribosyl transferase and orotidine-5'-decarboxylase)
742G 1575A
742C 1575G
742C 1575A
742 638G>C G213A
1424 1320C>T Silent
1575 1471A>G 3'
1603 1499delT 3'
J04031 J04031 172460 GEN-CB Methenyltetrahydrofolate cyclohydrolase
454G 969G 1614C 2011G
454A 969C 1614C 2011A
454A 969C 1614C 2011G
454G 969C 1614T 2011A
454G 969C 1614C 2011A
454G 969C 1614C 2011G
454 401G>A R134K
969 916C>G Q306E
1614 1561T>C Silent
2011 1958G>A R653Q
2335 2282C>T T761M
2358 2305C>T L769F
2368 2315G>A R772H
K02581 K02581 188300 GEN-CI Thymidine kinase 1
90C 279G 282G 772A 867G
90C 279A 282G 772A 867G
90T 279G 282G 772A 867G
90T 279G 282A 772A 867A
90C 279G 282A 772G 867A
90T 279A 282G 772A 867G

90T 279G 282A 772A 867G
90T 279G 282A 772G 867G
90T 279G 282G 772G 867G
90T 279G 282G 772G 867A
90C 279A 282G 772G 867A
90T 279A 282G 772G 867G
90C 279G 282G 772G 867A
90C 279G 282G 772G 867G
90T 279G 282A 772G 867A
90T 279A 282G 772G 867A
90 33C>T Silent
279 222G>A Silent
282 225G>A Silent
772 715A>G 3'
867 810G>A 3'

U09178 U09178 274270 GEN-HA Dihydropyrimidine Dehydrogenase

166C 577A 3925A 3937C
166C 577A 3925A 3937T
166C 577A 3925G 3937C
166T 577A 3925G 3937C
166T 577A 3925A 3937C
166T 577G 3925G 3937C
166T 577A 3925A 3937T
166 85T>C C29R
577 496A>G M166V
638 557A>G Y186C
1708 1627A>G I543V
3432 3351T>C 3'
3730 3649G>A 3'
3925 3844A>G 3'
3937 3856T>C 3'

U19720 U19720 600424 GEN-I1 Folate Transporter (SLC19A1)

175G 341C 791C 1067G 1337C 1997T 2582T 2617C 2652T
175G 341C 791T 1067G 1337C 1997T 2582G 2617T 2652T
175G 341C 791C 1067A 1337C 1997T 2582T 2617C 2652T
175A 341C 791T 1067G 1337C 1997C 2582G 2617T 2652T

175A 341C 791T 1067G 1337C 1997T 2582G 2617T 2652T
53 (-43)T>C 5'
175 80G>A R27H
341 246C>G Silent
791 696C>T Silent
1067 972G>A Silent
1337 1242C>A Silent
1997 1902T>C 3'
2100 2005^2006insG Frame
2582 2487T>G 3'
2617 2522C>T 3'
2652 2557T>C 3'
U77088 U77088 188250 GEN-K4 Thymidine kinase 2
1480C
1480T
1480 1472T>C 3'
X16396 X16396 None GEN-LC Methenyltetrahydrofolate dehydrogenase
1397G 1480A
1397A 1480A
1397A 1480G
1259 1244G>A 3'
1397 1382A>G 3'
1480 1465G>A 3'
X59543 X59543 180410 GEN-M2 Ribonucleotide reductase M1 polypeptide
1037A 2410G 2419A
1037C 2410A 2419G
1037C 2410A 2419A
1037C 2410G 2419A
1037A 2410A 2419A
1037A 2410A 2419G
1037 850C>A Silent
1749 1562G>A S521N
1846 1659C>T Silent
2410 2223G>A Silent
2419 2232A>G Silent
2717 2530T>A 3'

2724 2537^2538insT Frame
2882 2695A>C 3'
X59618 X59618 180390 GEN-M3 Ribonucleotide reductase M2 polypeptide
128G 189T 524C 1399T 1464G 1636C 1738C 2259T
128G 189G 524C 1399T 1464G 1636C 1738C 2259T
128 (-67)G>A 5'
189 (-6)T>G 5'
524 330C>G Silent
1399 1205T>A 3'
1464 1270G>A 3'
1636 1442C>T 3'
1738 1544C>T 3'
2259 2065T>C 3'
X90858 X90858 191730 GEN-NQ Uridine phosphorylase
309C
309T
309 (-44)C>T 5'
824 472G>A A158T
X17620 X17620 156490 GEN-20M Nucleoside Diphosphate Kinase A, partial
244 244G>T D82Y
488 488A>G H163R
L38928 L38928 604197 GEN-2PT Homo sapiens 5,10-methenyltetrahydrofolate synthetase mRNA, complete cds
617 604A>G T202A
U55206 U55206 601509 GEN-35Z Homo sapiens human gamma-glutamyl hydrolase (hGH) mRNA, complete cds
75T 150G 511C 703G 1161A
75T 150G 511T 703A 1161A
75T 150G 511C 703A 1161A
75T 150G 511C 703A 1161G
75 16T>C C6R
150 91G>A A31T
511 452C>T T151I
703 644A>G N215S
1161 1102A>G 3'
U81375 U81375 602193 GEN-3VO Human placental equilibrative nucleoside transporter 1 (hENT1) mRNA, complete cds
1466G 1989G 1996C 2045T
1466A 1989G 1996C 2045C

1466G 1989G 1996T 2045C
1466G 1989G 1996C 2045C
1466 1288G>A A430T
1989 1811G>A 3'
1996 1818C>T 3'
2045 1867T>C 3'
L11931 L11931 182144 GEN-4DT Human cytosolic serine hydroxymethyltransferase (SHMT) mRNA, complete cds
1444C 1541T
1444T 1541C
1444T 1541T
1444C 1541C
1444 1420C>T L474F
1523 1499C>G 3'
1541 1517C>T 3'
U29200 U29200 None GEN-4DU Nucleoside diphosphate kinase B promoter
1068T 1123A
1068A 1123G
1068T 1123G
1068 (-388)A>T Genomic
1123 (-333)G>A Genomic
X58965 X58965 156491 GEN-4DV Nucleoside Diphosphate Kinase B
634G
634A
634 562A>G 3'
CDA L27943 123920 GEN-4E4 Homo sapiens cytidine deaminase (CDA) mRNA, complete cds
552 435T>C Silent
DHFR J00140 126060 GEN-4E9 Human dihydrofolate reductase gene
721 679T>A 3'
829 787C>T 3'
U09806 U09806 236250 GEN-4FZ Human methylenetetrahydrofolate reductase mRNA, partial cds
120C 464T 519T 668C 1059C 1289A 1308C 1784G
120C 464T 519C 668T 1059C 1289A 1308T 1784G
120C 464T 519C 668C 1059C 1289C 1308T 1784G
120C 464T 519C 668C 1059C 1289A 1308C 1784G
120C 464T 519C 668C 1059C 1289A 1308T 1784G
120 120T>C Silent

464 464T>G M155R
519 519C>T Silent
668 668C>T A223V
1059 1059T>C Silent
1289 1289C>A 3'
1308 1308T>C 3'
1784 1784G>A 3'
M58602 M58602 131222 GEN-LUB Thymidine phosphorylase, promoter and genomic
124C 439G 1977A 2149G 2975G 3116G 3255A 3344T 4782G 5022C 5266G 5285C 5438T 5482C 5629G 5648C 5731A
124C 439G 1977G 2149G 2975G 3116G 3255A 3344C 4782G 5022C 5266G 5285C 5438T 5482C 5629G 5648T 5731G
124C 439G 1977G 2149A 2975G 3116G 3255A 3344T 4782G 5022T 5266G 5285C 5438T 5482C 5629G 5648C 5731G
124C 439G 1977G 2149G 2975G 3116G 3255A 3344T 4782G 5022C 5266G 5285C 5438T 5482C 5629G 5648C 5731A
124C 439G 1977G 2149G 2975G 3116G 3255A 3344T 4782G 5022T 5266G 5285C 5438A 5482C 5629G 5648C 5731G
124C 439G 1977G 2149G 2975G 3116G 3255A 3344T 4782G 5022T 5266G 5285C 5438T 5482C 5629G 5648C 5731G
124C 439G 1977G 2149G 2975G 3116G 3255A 3344T 4782G 5022T 5266G 5285C 5438T 5482C 5629A 5648C 5731G
124 124C>T Genomic
439 439G>A Genomic
1044 1044^insCT Genomic
1331 1331G>A Genomic
1977 1977G>A Genomic
2149 2149G>A Genomic
2467 2467A>G Genomic
2975 2975G>A Genomic
3116 3116G>T Genomic
3255 3255A>C Genomic
3344 3344T>C Genomic
4782 4782G>A Genomic
5022 5022T>C Genomic
5266 5266G>A Genomic
5285 5285C>G Genomic
5438 5438T>A Genomic
5482 5482C>T Genomic
5629 5629G>A Genomic
5648 5648C>T Genomic
5731 5731G>A Genomic
D00517 D00517 188350 GEN-LUC Thymidylate synthase, promoter

276C 321T 452G 491C 533C 624A 639A
276T 321T 452G 491C 533C 624C 639A
276T 321C 452G 491C 533C 624C 639A
276C 321T 452G 491C 533T 624C 639A
276C 321C 452G 491C 533T 624A 639A
276T 321T 452G 491C 533C 624A 639A
276T 321C 452A 491C 533C 624C 639A
276C 321T 452G 491C 533T 624A 639A
276T 321T 452G 491A 533C 624C 639G

276	276C>T	Genomic	
321	321T>C	Genomic	
452	452G>A	Genomic	
457	457^insC	Genomic	
491	491C>A	Genomic	
533	533T>C	Genomic	
624	624A>C	Genomic	
639	639A>G	Genomic	
655	655T>C	Genomic	
D00596	D00596 188350	GEN-LUD	Homo sapiens gene for thymidylate synthase, exons 1, 2, 3, 4, 5, 6, 7, complete cds
701	701A>C	Genomic	
716	716A>G	Genomic	
732	732T>C	Genomic	
1293	1293A>G	Genomic	
1322	1322C>G	Genomic	
1379	1379T>C	Genomic	
1590	1590C>T	Genomic	
1688	1688C>G	Genomic	
3083	3083G>A	Genomic	
3125	3125G>A	Genomic	
3212	3212C>T	Genomic	
3635	3635G>A	Genomic	
4256	4256G>A	Genomic	
13645	13645T>C	Genomic	
13751	13751C>A	Genomic	
13782	13782T>C	Genomic	
13806	13806T>C	Genomic	

13813 13813T>C Genomic
14479 14479A>G Genomic
14546 14546^insT Genomic
14585 14585C>T Genomic
14729 14729G>A Genomic
14795 14795G>A Genomic
15041 15041T>C Genomic
U24253 U24253 136510 GEN-LUE Human folylpolyglutamate synthetase (FPGS) gene, exons 5-11, and partial cds
1424A 1649G 2554A
1424C 1649G 2554G
1424C 1649A 2554A
1424C 1649G 2554A
1424 1424C>A Genomic
1649 1649G>A Genomic
2554 2554A>G Genomic
U24252 U24252 136510 GEN-LUF Folylpolyglutamate synthetase, promoter and exons 1-4
266G 527C 1037G 1139G 1217C 1647C 1955G 2017G 2189A 2282C
266G 527C 1037G 1139G 1217C 1647C 1955A 2017G 2189A 2282C
266G 527C 1037G 1139G 1217C 1647T 1955A 2017G 2189A 2282C
266G 527C 1037A 1139G 1217C 1647C 1955A 2017G 2189A 2282C
266T 527C 1037G 1139G 1217C 1647C 1955A 2017G 2189A 2282C
266G 527C 1037A 1139G 1217C 1647C 1955G 2017G 2189A 2282C
266T 527C 1037G 1139G 1217T 1647C 1955A 2017G 2189A 2282C
266T 527C 1037G 1139G 1217C 1647C 1955A 2017A 2189A 2282C
266G 527C 1037G 1139G 1217C 1647C 1955A 2017A 2189A 2282C
266G 527C 1037G 1139G 1217C 1647C 1955A 2017G 2189G 2282C
266G 527G 1037G 1139G 1217C 1647T 1955A 2017G 2189A 2282C
266 266G>T Genomic
527 527C>G Genomic
1037 1037A>G Genomic
1139 1139G>A Genomic
1217 1217C>T Genomic
1647 1647C>T Genomic
1955 1955G>A Genomic
2017 2017G>A Genomic
2189 2189A>G Genomic

2282 2282C>T Genomic
AF061655 AF061655 123920 GEN-LUJ Cytidine deaminase, promoter
575T 648T 771G 883G 1051A
575T 648T 771G 883G 1051C
575C 648T 771G 883A 1051A
575C 648C 771C 883A 1051A
575C 648T 771C 883G 1051A
575C 648T 771C 883A 1051A
575 575T>C Genomic
648 648T>C Genomic
771 771G>C Genomic
883 883G>A Genomic
941 941^insC Genomic
1051 1051A>C Genomic
K01612 K01612 None GEN-MT4 Dihydrofolate reductase, promoter
1120C 1124G 1135G 1229G 1678C
1120C 1124G 1135G 1229G 1678G
1120T 1124G 1135G 1229G 1678C
1120C 1124A 1135G 1229G 1678C
1120 1120C>T Genomic
1124 1124G>A Genomic
1135 1135A>G Genomic
1229 1229A>G Genomic
1678 1678C>G Genomic

Table 11

Variance Table

Hugo / Variance Start	GID	OMIM ID / Variance	VGX Symbol / CDS_Context	Description
D00596	D00596	188350	GEN-LUD	Homo sapiens gene for thymidylate synthase, exons 1, 2, 3, 4, 5, 6, 7, complete cds
235		235C>T	Genomic	
6652		6652A>G	Genomic	
15839		15839A>G	Genomic	
J03626	J03626	258900	GEN-C6	Uridine monophosphate synthetase (orotate phosphoribosyl transferase and orotidine-5'-decarboxylase)
742		638G>C	G213A	
1424		1320C>T	Silent	
J04031	J04031	172460	GEN-CB	Methenyltetrahydrofolate cyclohydrolase
3009		2956A>C	3'	
L38928	L38928	604197	GEN-2PT	Homo sapiens 5,10-methenyltetrahydrofolate synthetase mRNA, complete cds
617		604A>G	T202A	
U09178	U09178	274270	GEN-HA	Dihydropyrimidine Dehydrogenase
166		85T>C	C29R	
784		703C>T	R235W	
1682		1601G>A	S534N	
1708		1627A>G	I543V	
2275		2194G>A	V732I	
2738		2657G>A	R886H	
3064		2983G>T	V995F	
U09806	U09806	236250	GEN-4FZ	Human methylenetetrahydrofolate reductase mRNA, partial cds
668		668C>T	A223V	
1289		1289C>A	3'	
U73338	U73338	156570	GEN-69	Methionine Synthase
6750		6356G>A	3'	

Claims

What we claim is:

1. A method for selecting a treatment for a patient suffering from a condition or disease, comprising

determining whether cells of said patient contain at least one variance of a gene, wherein the presence or the absence of said variance in said cells is indicative of the effectiveness of said treatment for said condition or disease,

wherein said gene is a folate transport or metabolism gene or a pyrimidine transport or metabolism gene.

2. The method of claim 1, wherein said gene is selected from the group consisting of Folate receptor 1(α), Folate receptor (β), Folate receptor (γ), Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase.

3. The method of claim 1, wherein the presence of said at least one variance is indicative that said treatment will be effective for said patient.

4. The method of claim 1, wherein the presence of said variance is indicative that said treatment will be ineffective or contra-indicated for said patient.

5. The method of claim 1, wherein said at least one variance comprises a plurality of variances.

6. The method of claim 5, wherein said plurality of variances comprise a haplotype or haplotypes.

7. The method of claim 1, wherein said selecting a treatment further comprises identifying a compound differentially active on a form of said gene containing said at least one variance.

8. The method of claim 1, wherein said compound is selected from the group consisting of a reduced folate, a folate analog, folic acid, a fluoropyrimidine, a dihydropyrimidine dehydrogenase inhibitor, a cytidine analog, a pyrimidine analog, a ribonucletide reductase inhibitor, and a nucleotide/nucleoside uptake inhibitor.

9. The method of claim 1, wherein said selecting a treatment further comprises eliminating a treatment, wherein said presence or absence of said at least one variance is indicative that said treatment will be ineffective or contra-indicated.

10. The method of claim 1, wherein said treatment comprises a first treatment and a second treatment, said method comprising the steps of:

identifying a said first treatment effective to treat said disease or condition; and

identifying a said second treatment which reduces a deleterious effect of said first treatment.

11. The method of claim 1, wherein said selecting a treatment further comprises selecting the method of administration of a compound effective to treat said disease, wherein said presence or absence of said at least one variance is indicative of the appropriate method of administration for said compound.

12. The method of claim 11, wherein said selecting the method of administration comprises selecting a suitable dosage level or frequency of administration of a compound.

13. The method of claim 1, further comprising determining the level of expression of said gene or the level of activity of a protein containing a polypeptide expressed from said gene,

wherein the combination of the determination of the presence or absence of said at least one variance and the determination of the level of activty or the level of expression provides a further indication of the effectiveness of said treatment.

14. The method of claim 1, wherein said disease or condition is selected from the group consisting of cancer, proliferative skin diseases, autoimmune diseases, folate deficiency, cardiovascular disease, transplantation, and spina bifida.

15. The method of claim 1, wherein the detection of the presence or absence of said at least one variance comprises amplifying a segment of nucleic acid including at least one of said variances.

16. The method of claim 15, wherein said segment of nucleic acid is 500 nucleotides or less in length.

17. The method of claim 15, wherein said segment of nucleic acid is 100 nucleotides or less in length.

18. The method of claim 15, wherein said segment of nucleic acid is 45 nucleotides or less in length.

19. The method of claim 15, wherein said segment includes a plurality of variances.

20. The method of claim 1, wherein the detection of the presence or absence of said at least one variance comprises contacting nucleic acid comprising a variance site with at least one nucleic acid probe, wherein said at least one probe preferentially hybridizes with a nucleic acid sequence including said variance site and containing a complementary base at said variance site under selective hybridization conditions.

21. The method of claim 1, wherein the detection of the presence or absence of said at least one variance comprises sequencing at least one nucleic acid sequence.

22. The method of claim 1, wherein the detection of the presence or absence of said at least one variance comprises mass spectrometric determination of at least one nucleic acid sequence.

23. The method of claim 1, wherein the detection of the presence or absence of said at least one variance comprises determining the haplotype of a plurality of variances in a gene.

24. A method for selecting a method of treatment, comprising

comparing at least one variance in at least one gene in a patient suffering from a disease or condition with a list of variances in said at least one gene indicative of the effectiveness of at least one method of treatment, wherein said at least one gene is a folate transport or metabolism gene or a pyrimidine transport or metabolism gene.

25. The method of claim 24, wherein said gene is selected from the group consisting of Folate receptor 1(α), Folate receptor (β), Folate receptor (γ), Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase.

26. The method of claim 24, wherein said at least one variance comprises a plurality of variances.

27. The method of claim 24, wherein said list of variances comprises a plurality of variances.

28. The method of claim 24, wherein at least one said method of treatment comprises the administration of a compound effective against said disease or condition to a patient.

29. The method of claim 28, wherein said compound is selected from the group consisting of reduced folate, a folate analog, folic acid, a fluoropyrimidine, a dihydropyrimidine dehydrogenase inhibitor, a cytidine analog, a pyrimidine analog, a ribonucletide reductase inhibitor, and a nucleotide/nucleoside uptake inhibitor.

30. The method of claim 24, wherein the presence or absence of at least one variance in said gene is indicative that said treatment will be effective in said patient.

31. The method of claim 24, wherein the presence or absence of at least one variance in said gene is indicative that said treatment will be ineffective or contra-indicated.

32. The method of claim 24, wherein said treatment is a first treatment and the presence or absence of at least one variance in said gene is indicative that a second treatment will be beneficial to reduce a deleterious effect of said first treatment.

33. The method of claim 24, wherein said at least one method of treatment is a plurality of methods of treatment.

34. The method of claim 33, wherein said selecting comprises determining whether any of said plurality of methods of treatment will be more effective than at least one other of said plurality of methods of treatment.

35. The method of claim 24, wherein said disease is selected from the group consisting of cancer, proliferative skin diseases, autoimmune diseases, folate deficiency, cardiovascular disease, transplantation, and spina bifida.

36. A method for selecting a method of administration to a patient suffering from a condition or disease for a compound or compounds effective to treat said condition or disease, comprising the step of

determining whether at least one variance in a gene is present or absent in cells of said patient, wherein said presence or absence of said at least one variance is indicative of an appropriate method of administration for said compound, and

wherein said gene is a folate transport or metabolism or pyridine transport or metabolism gene.

37. The method of claim 36, wherein said gene is selected from the group consisting of Folate receptor 1(α), Folate receptor (β), Folate receptor (γ), Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase.

38. The method of claim 36, wherein said selecting a method of administration comprises selecting a dosage level or frequency or frequency of administration of said compound.

39. The method of claim 36, wherein said drug is selected from the group consisting of reduced folate, a folate analog, folic acid, a fluoropyrimidine, a dihydropyrimidine dehydrogenase inhibitor, a cytidine analog, a pyrimidine analog, a ribonucletide reductase inhibitor, and a nucleotide/nucleoside uptake inhibitor.

40. The method of claim 36, wherein said disease is selected from the group consisting of cancer, proliferative skin diseases, autoimmune diseases, folate deficiency, cardiovascular disease, transplantation, and spina bifida .

41. A method for selecting a patient for administration of a method of treatment, comprising

comparing the presence or absence of at least one variance in a gene in cells of a patient suffering from a disease or condition with a list of variances in said gene, wherein the presence or absence of said at least one variance in said cells is indicative that said treatment will be effective in said patient; and

determining whether said patient will receive said method of treatment based on the presence or absence of said at least one variance in said cells,

wherein said gene is a folate transport or metabolism gene or a pyrimidine transport or metabolism gene.

42. The method of claim 41, wherein said gene is selected from the group consisting of Folate receptor 1(α), Folate receptor (β), Folate receptor (γ), Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

43. The method of claim 41, wherein said method of treatment comprises administration of a compound effective against said disease or condition.

44. The method of claim 43, wherein said disease is selected from the group consisting of reduced folate, a folate analog, folic acid, a fluoropyrimidine, a dihydropyrimidine dehydrogenase inhibitor, a cytidine analog, a pyrimidine analog, a ribonucletide reductase inhibitor, and a nucleotide/nucleoside uptake inhibitor .

45. The method of claim 41, wherein said determining comprises assigning said patient to a group to receive said method of treatment or to a control group.

46. A method for identifying the presence or absence of at least one form of a gene in cells of an individual, comprising the steps of:

a) determining the presence or absence of at least one variance in said gene in said cells, wherein said gene is a folate transport or metabolism or pyrimidine transport or metabolism gene.

47. The method of claim 46, wherein said gene is selected from the group consisting of Folate receptor 1(α), Folate receptor (β), Folate receptor (γ), Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

48. The method of claim 46, wherein said individual suffers from a disease or condition.

49. The method of claim 46, wherein the presence or absence of said at least one variance is indicative of the effectiveness of a therapeutic treatment in a patient having cells containing said at least one variance.

50. The method of claim 46, wherein said determining comprises amplifying a segment of nucleic acid including a site of at least one of said at least one variance.

51. The method of claim 46, wherein said determining comprises contacting a nucleic acid sequence containing a variance site corresponding to a said variance with a probe which specifically binds under selective binding conditions to a nucleic acid sequence comprising at least one said variance.

52. The method of claim 46, wherein the detection of the presence or absence of said at least one variance comprises sequencing at least one nucleic acid sequence.

53. The method of claim 46, wherein the detection of the presence or absence of said at least one variance comprises mass spectrometric determination of at least one nucleic acid sequence.

54. The method of claim 46, wherein the detection of the presence or absence of said at least one variance comprises determining the haplotype of a plurality of variances in a gene.

55. A pharmaceutical composition comprising

a compound which has a differential effect in patients having at least one copy of a particular form of a gene, wherein said gene is a folate transport or metabolism gene or a pyrimidine transport or metabolism gene; and

a pharmaceutically acceptable carrier or excipient or diluent,

wherein said composition is adapted to be preferentially effective to treat a patient with cells comprising a form of said gene comprising at least one variance.

56. The composition of claim 55, wherein said gene is selected from the group consisting of Folate receptor 1(α), Folate receptor (β), Folate receptor (γ), Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-

drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

57. The compositon of claim 55, wherein said patient suffers from a disease or condition selected from the group consisting of cancer, proliferative skin diseases, autoimmune diseases, folate deficiency, cardiovascular disease, transplantation, and spina bifida .

58. The pharmaceutical composition of claim 55, wherein said pharmaceutical composition is subject to a regulatory limitation restricting the use of said pharmaceutical composition to patients having at least one copy of a form of a gene comprising at least one variance.

59. The pharmaceutical composition of claim 55, wherein said pharmaceutical composition is subject to a regulatory limitation indicating said pharmaceutical composition is not to be used in patients having at least one copy of a form of a gene comprising at least one variance.

60. The pharmaceutical composition of claim 55, wherein said pharmaceutical composition is packaged, and the packaging includes a label or insert restricting the use of said pharmaceutical composition to patients having at least one copy of a form of a gene comprising at least one variance.

61. The pharmaceutical composition of claim 55, wherein said pharmaceutical composition is packaged, and said packaging includes a label or insert requiring the use

of a test to determine the presence or absence of at least one variance in cells of a said patient.

62. A probe which specifically binds under selective binding conditions to a nucleic acid sequence comprising at least one variance in a gene selected from the group consisting of Folate receptor 1(α), Folate receptor (β), Folate receptor (γ), Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

63. The probe of claim 62, wherein said probe comprises a nucleic acid sequence 500 nucleotide bases or fewer in length.

64. The probe of claim 62, wherein said nucleic acid sequence is 100 or fewer nucleotide bases in length.

65. The probe of claim 62, wherein said nucleic acid sequence is 25 or fewer nucleotide bases in length.

66. The probe of claim 62, wherein said probe comprises DNA.

67. The probe of claim 62, wherein said probe comprises DNA and at least one nucleic acid analog.

68 The probe of claim 62, wherein said probe comprises peptide nucleic acid (PNA

69. The probe of claim 62, further comprising a detectable label.

70. The probe of claim 69, wherein said detectable label is a fluorescent label.

71. A method for determining a genotype of an individual, comprising analyzing at least one nucleic acid sequence from cells of said individual using mass spectrometric analysis,

wherein said nucleic acid sequence is a portion of a folate transport or metabolism gene or pyrimidine transport or metabolism gene or a complementary sequence.

72. The method of claim 71, wherein said analyzing a nucleic acid sequence comprises determining the presence or absence of a variance in said gene.

73. The method of claim 71, wherein said analyzing a nucleic acid sequence comprises determining the nucleotide sequence of said at least one nucleic acid sequence.

74. The method of claim 71, wherein said at least one nucleic acid sequence is 500 nucleotides or less in length.

75. The method of claim 71, wherein said at least one nucleic acid sequence comprises at least one variance site in said gene.

76. An isolated, purified or enriched nucleic acid sequence of 15 to 500 nucleotides in length, comprising at least one variance, wherein said sequence has the base sequence of a portion of an allele of a gene selected from the group consisting of Folate receptor 1(α), Folate receptor (β), Folate receptor (γ), Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate

synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase or a sequence complementary thereto.

77. The nucleic acid sequence of claim 76, wherein said nucleic acid sequence is 15 to 100 nucleotide bases in length.

78. The nucleic acid sequence of claim 76, wherein said nucleic acid sequence sequence is 15 to 25 nucleotide bases in length.

79. A method for determining whether a compound has differential effects on cells containing at least one different form of a folate transport or metabolism or pyridine transport or metabolism gene, comprising the steps of:

contacting a first cell and a second cell with said compound, wherein said first cell and said second cell differ in the presence or absence of at least one variance in said gene; and

determining whether the response of said first cell and said second cell to said compound differ, wherein the difference in said response is due to the presence or absence of said at least one variance.

80. The method of claim 79, wherein said gene is selected from the group consisting of Folate receptor 1(α), Folate receptor (β), Folate receptor (γ), Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-

iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

81. The method of claim 79, wherein at least one of said first cell and said second cell are contacted *in vivo*.

82. The method of claim 79, wherein at least one of said first cell and said second cell are contacted *in vitro*.

83. The method of claim 81, wherein at least one of said first cell and said second cell is contacted *in vivo* in a plurality of patients suffering from a disease or condition

84. A method of treating a patient suffering from a condition or disease, comprising the steps of:

a) determining whether cells of said patient contain a form of a gene which comprises at least one variance, wherein the presence or absence of said at least one variance is indicative that a treatment will be effective in said patient; and

b) administering said treatment to said patient.

85. The method of claim 84, wherein said gene is a folate transport or metabolism gene or a pyrimidine transport or metabolism gene. .

86. The method of claim 84, wherein said gene is selected from the group consisting of Folate receptor 1(α), Folate receptor (β), Folate receptor (γ), Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase,

Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formyltetrahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate formiminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

87. The method of claim 84, wherein said disease is selected from the group consisting of cancer, proliferative skin diseases, autoimmune diseases, folate deficiency, cardiovascular disease, transplantation, and spina bifida .

88. The method of claim 84, wherein the presence of said at least one variance is indicative that said treatment will be effective in said patient.

89. The method of claim 88, wherein said treatment comprises the administration of a compound preferentially active for said condition or disease in a said patient having said at least one variance in said gene.

90. The method of claim 89, wherein said compound is selected from the group consisting of reduced folate, a folate analog, folic acid, a fluoropyrimidine, a dihydropyrimidine dehydrogenase inhibitor, a cytidine analog, a pyrimidine analog, a ribonucletide reductase inhibitor, and a nucleotide/nucleoside uptake inhibitor .

91. The method of claim 84, wherein the presence of said at least one variance in said gene is indicative of an appropriate dosage or frequency of administration of a compound in said treatment.

92. A method of treating a patient suffering from a disease or condition, comprising the steps of:

a) comparing the presence or absence of at least one variance in at least one gene in cells of a patient suffering from a disease or condition with a list of variances in said at least one gene indicative of the effectiveness of at least one method of treatment;

b) selecting a method of treatment from said at least one method of treatment, wherein the presence or absence of at least one of said at least one variance is indicative that said method of treatment will be effective in said patient; and

c) administering said method of treatment to said patient.

93. The method of claim 92, wherein said at least one gene comprises a folate transport or metabolism or pyrimidine transport or metabolism gene.

94. The method of claim 92, wherein said gene is selected from the group consisting of Folate receptor 1(α), Folate receptor (β), Folate receptor (γ), Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

95. The method of claim 92, further comprising determining the presence or absence of said at least one variance in cells of said patient.

96. The method of claim 92, wherein said at least one variance comprises a plurality of variances.

97. The method of claim 92, wherein said list of variances comprises a plurality of variances.

98. The method of claim 97, wherein said plurality of variances comprises a haplotype or haplotypes.

99. The method of claim 92, wherein said method of treatment comprises the administration of a compound effective against said disease or condition.

100. The method of claim 92, wherein said treatment is a first treatment and the presence or absence of at least one variance in said gene is indicative that a second treatment will be beneficial to reduce a deleterious effect of said first treatment.

101. The method of claim 92, wherein said at least one method of treatment is a plurality of methods of treatment.

102. The method of claim 92, wherein said disease or condition is selected from the group consisting of cancer, proliferative skin diseases, autoimmune diseases, folate deficiency, cardiovascular disease, transplantation, and spina bifida .

103. A method of treating a patient suffering from a disease or condition, comprising the steps of:

a) comparing the presence or absence of at least one variance in at least one gene in cells of a patient suffering from a disease or condition with a list of variances in said at least one gene indicative of the effectiveness of at least one method of treatment;

b) eliminating a method of treatment from said at least one method of treatment, wherein the presence or absence of at least one of said at least one variance is indicative that said method of treatment will be ineffective or contra-indicated in said patient;

c) selecting an alternative method of treatment effective to treat said disease or condition; and

e. administering said alternative method of treatment to said patient.

104. The method of claim 103, further comprising determining the presence or absence of said at least one variance in cells of said patient.

105. The method of claim 103, wherein said at least one gene comprises a folate transport or metabolism or pyrimidine transport or metabolism gene.

106. The method of claim 103, wherein said gene is selected from the group consisting of Folate receptor 1(α), Folate receptor (β), Folate receptor (γ), Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

107. A method for producing a pharmaceutical composition, comprising the steps of:

a) identifying a compound which has differential activity against a disease or condition in patients having at least one variance in a gene;

b) compounding said pharmaceutical composition by combining said compound and a pharmaceutically acceptable carrier or excipient or diluent in manner adapted to be preferentially effective in patients having said at least one variance.

108. A method for producing a pharmaceutical agent, comprising the steps of:

a) identifying a compound which has differential activity against a disease or condition in patients having at least one variance in a gene;

b) synthesizing said compound in an amount sufficient to provide a pharmaceutical effect in a patient suffering from said disease or condition.

109. A method for determining whether a variance in a gene provides variable patient response to a method of treatment for a disease or condition, comprising the steps of:

determining whether the response of a first patient or set of patients suffering from a disease or condition differs from the response of a second patient or set of patients suffering from said disease or condition;

determining whether the presence or absence of at least one variance in at least one folate transport or metabolism gene or pyrimidine transport or metabolism gene differs between said first patient or set of patient and said second patient or set of patients;

wherein correlation of said presence or absence of at least one variance and the response of said patient to said treatment is indicative that said at least one variance provides variable patient response.

110. The method of claim 109, further comprising identifying at least one variance in a said gene.

111. The method of claim 109, wherein a plurality of pairwise comparisons of treatment response and the presence or absence of at least one variance are performed for a plurality of patients.

112. The method of claim 109, wherein said determining whether the presence or absence of at least one variance in at least one gene comprises comparing the response of at least one patient homozygous for said at least one variance with at least one patient homogyzous for the alternative form of said at least one variance.

113. The method of claim 109, wherein said determining whether the presence or absence of said at least one variance in at least one gene comprises comparing the response of at least one patient heterogyzous for said at least one variance with the response of at least one patient homozygous for said at least one variance.

114. The method of claim 109, wherein it is previously known that patient response to said method of treatment is variable.

115. The method of claim 109, wherein said gene is selected from the group consisting of Folate receptor 1(α), Folate receptor (β), Folate receptor (γ), Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase.

Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

116. The method of claim 109, wherein said disease or condition is selected from the group consisting of cancer, proliferative skin diseases, autoimmune diseases, folate deficiency, cardiovascular disease, transplantation, and spina bifida .

117. The method of claim 109, wherein said method of treatment comprises administration of a compound effective to treat said disease or condition.

118. A kit for determination of the presence or absence of at least one sequence variance in a gene identified in any of Tables 2, 6, and 8.

119. The kit of claim 118, wherein said variance is listed in any of Tables 3, 4, 10, and 11.

Abstract of the Disclosure

The present disclosure describes the use of genetic variance information for folate transport or metabolism genes or pyrimidine transport or metabolism genes in the selection of effective methods of treatment of a disease or condition. The variance information is indicative of the expected response of a patient to a method of treatment. Methods of determining relevant variance information and additional methods of using such variance information are also described.

Figure 1

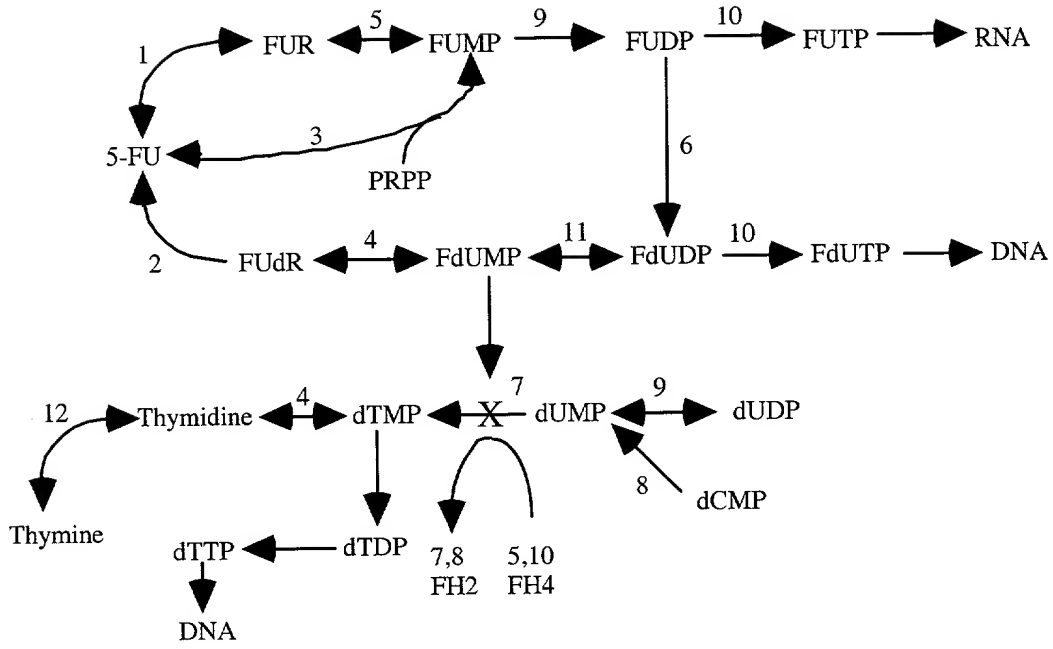
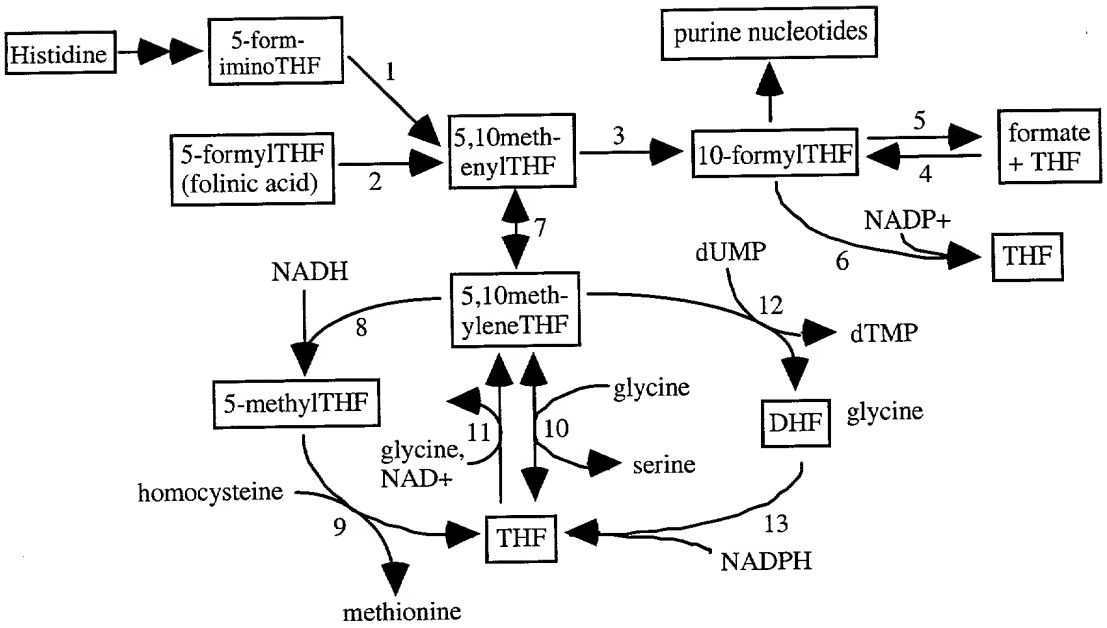

FUR
FUMP
FUDP
FUTP
RNA
5-FU
PRPP
FUdR
FdUMP
FdUDP
FdUTP
DNA
Thymidine
dTMP
dUMP
dUDP
dCMP
Thymine
dTTP
dTDP
DNA
7,8 FH2
5,10 FH4
1
2
3
4
5
6
7
8
9
10
11
12

Figure 2


Histidine
5-form-iminoTHF
1
purine nucleotides
5-formylTHF (folinic acid)
2
5,10meth-enylTHF
3
10-formylTHF
5
4
formate + THF
NADP+
6
THF
7
NADH
dUMP
5,10meth-yleneTHF
8
12
dTMP
5-methylTHF
glycine
11
10
glycine, NAD+
serine
DHF
glycine
homocysteine
9
THF
13
NADPH
methionine